A PHARMACOLOGICAL STUDY OF TRANSMISSION IN A SYMPATHETIC GANGLION

Thesis

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Doctor of Philosophy
in the University of Edinburgh

by

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PREFACE

This thesis presents an account of three related investigations on synaptic transmission in the sympathetic ganglion of the frog. Part I is concerned with the mode of action of the transmitter, its identity and the modifications in transmission caused by various drugs. Parts II and III are concerned with the spontaneous and the evoked release of the transmitter from the presynaptic nerve.

A brief report of part of the work described in Part III has been published (Blackman, Ginsborg and Ray, 1962).

I N D E X

PART I

INTRODUCTION 1

METHODS 7

Preparation
Stimulation 8
Microelectrodes 8
Recording apparatus 9
Time constant 9
Recording 11
Iontophoresis 11
Replacement of bath fluid 12
Method of impalement 13

RESULTS 17

1. Innervation 17
2. Mode of action of the synaptic transmitter 19
   Comparison of antidromic and orthodromic responses
   Interaction of effects of preganglionic and antidromic stimulation 20
   Analysis on the basis of a model circuit ... 22
3. The after-hyperpolarization 25
   Effect of changes in the external ionic concentration
   Effects of repetitive stimuli ... 26
   Effects of changing K^+ on the orthodromic response 27
4. Action of blocking agents 29
   Non-depolarizing blocking agents
   Depolarizing blocking agents ... 30
   Anticholinesterases 31
5. Iontophoretic application of acetylcholine 32

DISCUSSION 35

Identity of the synaptic transmitter ... 35
Mode of action of the transmitter ... 40

PART II

INTRODUCTION 43

METHODS 44

RESULTS 46

1. Spontaneous synaptic potentials
   Frequency
   Amplitude 47
   Time course and conductance ... 48
2. Factors modifying the frequency ... 50
   Effects of potassium

DISCUSSION 54

PART III

INTRODUCTION 57

RESULTS

1. Fluctuations of responses in normal Ringer 59
2. Fluctuations after depression of output of acetylcholine to low levels ... 60
3. Fluctuations at higher levels of output 64
4. Fluctuations in the presence of curare 66

DISCUSSION 68

APPENDIX

Analysis from failures 71
Analysis in the absence of failures ... 72
Analysis when probability of release is high... 75
Sampling errors 76

REFERENCES 77

FIGURES

	between pp.
Figure 1	7 - 8
2	9 - 10
3a,3b,4	10 - 11
5	15 - 16
6	17 - 18
7	19 - 20
8,9	20 - 21
10	22 - 23
11	23 - 24
12,13	25 - 26
14,15	26 - 27
16	27 - 28
17	28 - 29
18,19,20	29 - 30
21A	30 - 31
21,22A	31 - 32
22	33 - 34
23,24,25	46 - 47
26,27,27A	47 - 48
28	49 - 50
29,30	52 - 53
31	59 - 60
32	60 - 61
33,34	61 - 62
35	62 - 63
36,36	65 - 66
37	66 - 67

TABLES

Table I	15 - 16
II,III,IV	19 - 20
V	26 - 27
VI	46 - 47
VII	49 - 50
VIII	61 - 62
IX	65 - 66
X	66 - 67

INTRODUCTION

In 1914 Dale showed that acetylcholine stimulated mammalian ganglia. This observation may be regarded as the starting point of the work which culminated in the proof of cholinergic transmission. The presence of acetylcholine in mammalian sympathetic ganglion was first reported by Witanowski (1925) and later confirmed by Chang and Gaddum (1933), who in fact first suggested that acetylcholine might be the transmitter. That acetylcholine was released from preganglionic sympathetic fibres and that its injection had similar effects as those of preganglionic stimulation were first shown by Feldberg and Minz (1933) and Feldberg, Minz and Tsudzimura (1934) at the adrenal medulla in the cat and similar results were rapidly extended to the superior cervical ganglion by Feldberg and Gaddum (1934) using the technique developed by Kibjakow (1933). The release of acetylcholine did not occur on antidromic stimulation (Feldberg and Vartiainen, 1935) or after preganglionic denervation (Brown and Feldberg, 1936); in the absence of an anticholinesterase, acetylcholine was not obtained in the perfusate although a comparable increase in the output of choline was found (Brown and Feldberg, 1937; cf. Perry, 1953).

These findings strongly suggested that transmission in the mammalian ganglia was mediated by acetylcholine, but although the results which have been described were not disputed chemical transmission at sympathetic synapses was not generally accepted even as late as 1944 (Eccles, 1944).

The difficulty arose as a result of the interpretation of records of the electrical activity of the sympathetic ganglion obtained with extracellular electrodes. The first description of the action potential of a sympathetic ganglion was reported by Eccles (1935, 1936: superior cervical ganglion of the cat), and further examples from different ganglia were reported by Obrador and Odoriz (1936: the fifth lumbar ganglion of the cat), Whitteridge (1937: ciliary ganglion of the cat; cf. Perry and Talesnik, 1953), Lloyd (1937: inferior mesenteric ganglion of the cat), Bronk (1939: stellate ganglion of the cat), Lorente de Nó and Laporte (1950: superior cervical ganglion of the turtle), R.M. Eccles (1952: superior cervical ganglion of the rabbit) and Pascoe (1956: superior cervical ganglion of the rat). All the records obtained were in agreement, the initial phase of the activity after preganglionic stimulation consisting of a spike which was followed by a negative and a positive phase. The positive phase was also present in action potentials on 'antidromic'

stimulation and could therefore be regarded as a consequence of the action potential, and not attributable directly to the effect of the synaptic transmitter (Eccles, 1936). The negative wave following the spike was generally admitted to be due to the action of the acetylcholine released from the nerve terminals. It was enhanced by eserine (Rosenblueth and Simeone, 1938) and reduced by nicotine (Eccles, 1935; Lloyd, 1937; Whitteridge, 1937). The spike however did not appear to arise from the synaptic potential, whose full time course could be revealed by application of curare (Eccles, 1943; Lorente de Nó and Laporte, 1950), but it appeared to arise with a shorter latency.

From these and other results (Eccles, 1944), it was inferred that there were two components of activity at the synapse, the first being the sub-synaptic currents generated by the presynaptic nerve terminals, which generated the spike; the second was that underlying the negative wave, and not normally involved in the generation of the action potential. It was accepted that the second component was due to the action of acetylcholine. The complex records obtained with external electrodes are probably to be partly attributed to asynchrony in the response of different cells and partly to 'diphasic' artefacts. Although the "electrical theory" of transmission

at synapses was subsequently abandoned (on the basis of observations made on motoneurones of the mammalian central nervous system (Brock, Coombs and Eccles, 1952), the discrepancy between the time course of the synaptic potential and the action potential was not fully resolved until intracellular electrodes were used to record the action potential in response to preganglionic stimulation. It was then clearly shown that the spike did arise from the synaptic potential (R.M. Eccles, 1955; Nishi and Koketsu, 1960) and that the response to preganglionic stimulation differed in a characteristic way from the response to direct or antidromic stimulation (R.M. Eccles, 1956; Nishi and Koketsu, 1960). It has been frequently suggested that the difference is due to the concurrent action of the synaptic transmitter during the 'orthodromic action potential'. One of the objects of this investigation was to subject this idea to a direct test.

The mode of action underlying the production of the synaptic potential by the transmitter remained speculative until it was shown by Nishi and Koketsu (1960) that the synaptic transmitter in the frog produced a relatively non-specific change in the permeability of the post-synaptic membrane. Its action is therefore similar to that of the neuromuscular transmitter in skeletal muscle(see Katz, 1962).

Experiments which confirm this result are described in section 3.

Since the demonstration that acetylcholine depolarizes ganglion cells in mammals (Paton and Perry, 1953; Pascoe, 1956), the evidence that acetylcholine is the transmitter in the mammalian ganglion may be regarded as virtually complete (cf. Paton and Perry, 1953). Although it has been suggested that acetylcholine might not be the synaptic transmitter at amphibian ganglia (see Malcolm, 1949), no evidence is available on the identity of the synaptic transmitter in the frog, and another of the objects of the present investigation was to test the possibility that the transmitter was acetylcholine.

Paton and Perry (1953) also investigated the action of blocking agents on the superior cervical ganglion of the cat and showed that they could be grouped into two classes, depolarizing blocking agents and non-depolarizing blocking agents. Among the depolarizing drugs are nicotine and tetramethylammonium, and among the non-depolarizing agents are (+)-tubocurarine, decamethonium, hexamethonium and pentamethonium. These results are consistent with the classical effects of stimulation followed by block on application of nicotine (Langley and Dickinson, 1890) and block without stimulation with curare (1890 a). The third aim of the present work was to test whether this classification

was also true for the frog.

The work to be described is based on the analysis of intracellular responses from cells in the sympathetic ganglia of the frog. As has already been mentioned, experiments were made to provide further evidence on the mode of action of the synaptic transmitter and these are described in section 2. The experiments on the action of blocking drugs and the action of acetylcholine, applied to cells by iontophoresis are described in sections 4 and 5. In addition, the membrane changes underlying the positive phase which follows the spike have been examined and are described in section 3.

METHODS

Preparation: The frogs were decapitated and the spinal cord destroyed. The sympathetic chain, together with the dorsal aorta and the eighth, ninth and the tenth spinal nerves (numbered according to Ecker and Wiedersheim, 1899) were dissected out (Fig. 1). The preparation was gently stretched out under Ringer's fluid with the help of ligatures and part of the sheath around each of the lumbar ganglia (8th to 10th) was removed. Care was taken not to damage the rami communicantes and the inter-ganglionic chain. The connective tissue attaching the chain to the aorta was left intact.

The sympathetic chain above the seventh ganglion was cut away and the preparation transferred to a 'perspex' dish, which was provided with a central platform. The preparation was gently stretched out so that the cleaned ganglia were resting on the platform. The dish was filled with Ringer's fluid until the ganglia were just covered. The volume of the bath was about 15 ml.

The preparation was illuminated from below with the help of a low-power dark-ground condenser (Cooke, Troughton and Simms, N.A. 0.7-0.8), and a Zeiss binocular dissecting microscope (magnification 80X) was used to observe the cells during impalement

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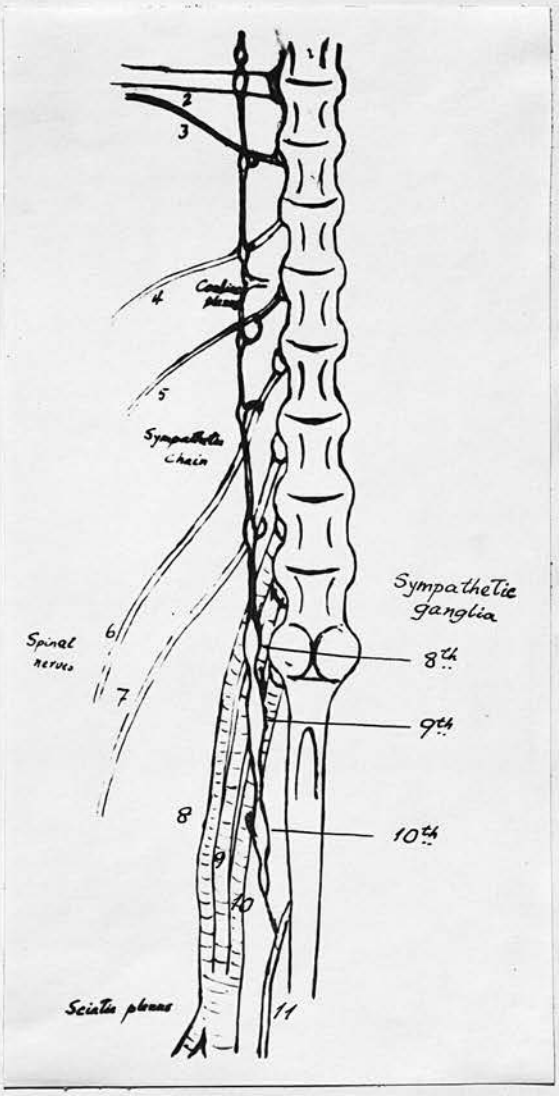


Fig. 1. Diagram showing the spinal nerves and the sympathetic chain on one side of the vertebral column.

with microelectrodes (Fig. 2).

Most of the experiments were performed at temperatures of 18° -20° C.

The Ringer's fluid used had the ionic composition of Na^+ -114.4 mM, K^+-2.0 mM, Ca^{++} 1.8 mM, Cl^-- 117.6 mM, HCO_3^--2.4 mM and glucose -1.11mM (cf. Nishi and Koketsu, 1960). Alterations in the composition were made when necessary and are described in the appropriate sections.

Stimulation: Capillary electrodes, described by Furshpan and Potter (1959), were used for stimulating the preparation. A short length (3-4 mm) of the sympathetic chain between the seventh and the eighth ganglia was sucked into one of the capillaries, and the sciatic plexus, along with nearby branches of the eighth and the eleventh spinal nerves, was sucked into the other capillary. The internal diameter of the capillaries was such that they fitted the nerves moderately tightly. Gentle suction was maintained in the capillaries to hold the nerves in position. Platinum wires dipping into the fluid inside the capillaries formed the anodes of the stimulating electrodes and platinum loops around the tips, the cathodes. The electrodes were connected to square-wave stimulators through isolating transformers.

Microelectrodes: Microelectrodes were pulled by machine (Frank and Fuortes, 1955) from 'Phoenix' or pyrex glass (2 mm o.d., 1 mm i.d.) and usually filled

with 3M KCl, or sometimes with 5M NaCl, by boiling under reduced pressure. Some electrodes were filled by the method described by Caldwell and Downing (1955). Electrodes having a resistance of 30 to 40 MΩ were used. The resistances were determined as in Fig. 3a.

Recording apparatus: Conventional recording arrangements were used (Fig. 2). The first stage consisted of a double-sided cathode-follower using ME 1400 valves. The input valve was checked for low grid current in normal operating conditions by measuring the voltage drop across a high resistance (500 MΩ) connected between the grid and the earth. The selected valve had a grid current of 4×10^{-12} A. The chlorided silver wire connecting the microelectrode with the input valve was kept short, and the microelectrode shielded, the shield being connected to the cathode to reduce the input capacitance of the system (Nastuk and Hodgkin, 1950). The bath electrode consisted of a chlorided silver wire embedded in agar-Ringer and was connected through a calibrator to the earthed side of the cathode follower. The output of the cathode follower was led through a short shielded cable to the inputs of one of the differential amplifiers of a Tektronix oscilloscope (model 502).

Time constant: The time constant of the system was determined by applying a square pulse between

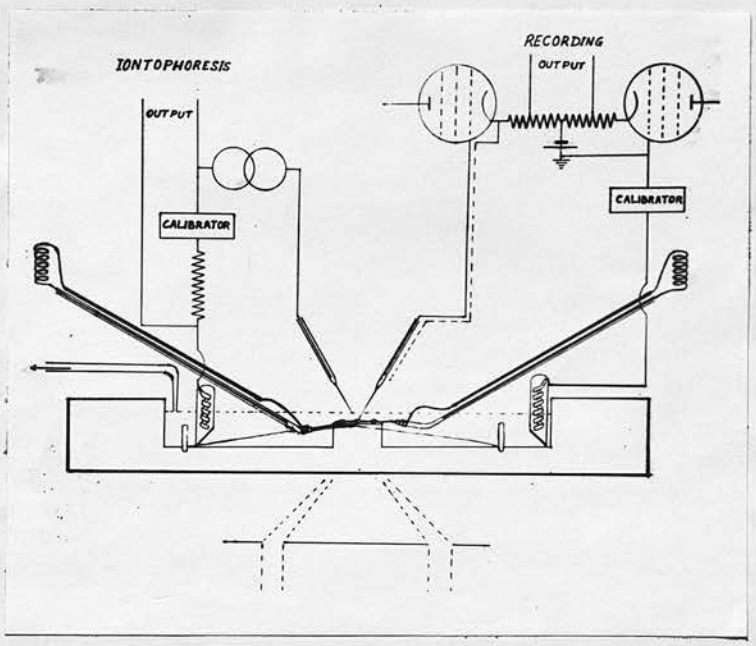


Fig. 2. The general arrangement of the preparation.

the bath electrode and earth (Fig. 3b). Since the capacitance of the microelectrode to earth depends upon the length of the tapered portion dipping in the bath (Nastuk and Hodgkin, 1950), the length actually dipping in the bath was carefully adjusted to that used for actual experiments. The capacitance to earth of the system without the microelectrode was found to be 2 pF. The capacitance of the tip was found to be 1 pF per mm dipping in the Ringer's fluid (cf. Fatt, 1961). The time constant of the system, using a microelectrode of 30 MΩ resistance dipping 2 mm below the surface of the Ringer's fluid, was found to be 120 μsec. The depth of the fluid above the ganglion was kept low, of the order of 1-2 mm, to keep the time constant low.

The time-course of the action potential was modified by the input characteristics of the recording system. This did not cause any serious error in the amplitudes of the responses. A graphical method was used to correct for the distortions (Lucas, 1912; Rushton, 1937; see Donaldson, 1958). In Fig. 4 the antidromic response of one of the cells was corrected assuming an input time-constant of 200 μsec. This was probably an overestimate of the actual input time-constant; it will be noticed that the amplitude of the spike increased from 94 mV to 95.5 mV. The rise-time of the spike was altered from 2.1 ms to 1.8 ms. These changes were not

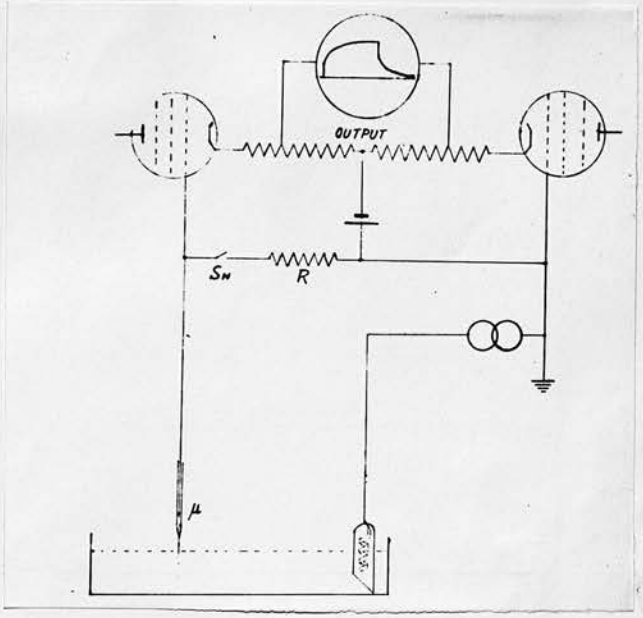


Fig. 3 (a). Measurement of microelectrode resistance. A square pulse is applied to the bath electrode and an output of V_1 (the plateau) observed. With the switch (Sw) shut the output falls to V_2. The microelectrode resistance is given by $R \times \frac{V_1 - V_2}{V_2}$

Fig. 3 (b). Measurement of input time constant. A square pulse is applied to the bath electrode. The time constant is determined from the exponential part of the output, which rises and falls with the time constant T.

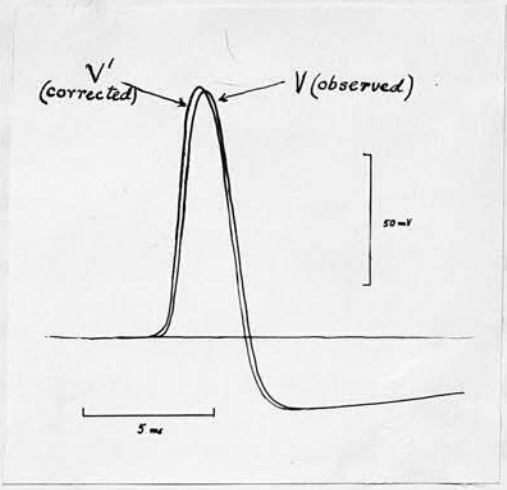


Fig. 4. Correction for input time constant. Cell 39. The antidromic action potential was reconstructed assuming an input time constant of 200 μsec, from the equation $V' = V + \tau \frac{dV}{dt}$ where V' is the true potential difference at any point giving rise to V, the observed potential, $\frac{dV}{dt}$ the rate of change of potential and τ the input time constant.

large currents (of the order of several microamperes) without distorting the shape of the pulse were selected. They were filled with 2.5 M acetylcholine chloride by replacement of KCl and stored in a refrigerator.

The apparatus for producing the pulses had an arrangement to provide steady potentials - this prevented diffusion of cations from the micropipettes when the inside was made negative with respect to the outside.

The general arrangement is illustrated (Fig. 2). The current pulse was monitored by measuring the potential drop across a bank of resistors of known value. The circuit was completed through the bath by means of a silver/silver chloride electrode embedded in Ringer-agar and a calibrator.

During use of the iontophoresis device the bath was disconnected from earth at the recording bath electrode, the earth being at the micropipette terminal.

The potential difference across the monitor resistance was displayed on the second beam of the Tektronix oscilloscope.

Replacement of the bath fluid: When necessary, the bathing fluid could be totally replaced without appreciable change in the level. This was done by

adding fluid to the bath from large syringes and at the same time applying suction to the surface from a water pump. It was necessary to add about four times the bath volume of fresh fluid. The suction was applied via a siliconed trap to avoid an earth loop. About two or three minutes were necessary to change the fluid, the disturbance being enough to ensure thorough mixing, but sometimes not violent enough to dislodge the microelectrode from inside the cell.

Drugs were added from small syringes through polythene cannulae. Care was taken to draw up the bath fluid and expel it several times through the cannulae to effect mixing.

Method of impalement: The preparation was stimulated antidromically at a rate of 1 to 5 per second. The microelectrode was brought near the preparation till the trace on the oscilloscope face showed movement due to alterations in the 'tip potential' of the microelectrode (cf. Ito, 1957).

The electrode was then lowered gradually into the preparation. At this stage, it was occasionally possible to obtain externally recorded action potentials. These usually consisted of a sharp positive spike followed by a slower and smaller negative potential.

On lowering the electrode further, the externally recorded complex described above changed into a slower, larger and entirely positive response. It was rarely possible to continue the downward movement of the microelectrode into the cell. More often, a sharp tap on the table was all that was necessary to cause the microelectrode to enter a cell.

The sequence of events after entry was essentially similar whether the microelectrode was lowered in gradually or inserted by tapping on the table. A steady negative potential, the magnitude varying widely from cell to cell, was often seen. The antidromic response at this stage was usually small and slow. On waiting, the negative potential often increased and the amplitude and the rate of rise of the response also increased. On other occasions, the response deteriorated rapidly and the electrode was moved elsewhere and the process of entry repeated. Occasionally, long trains of spontaneous potentials arose out of a fluctuating base line; these potentials were possibly generated from a damaged membrane.

It was sometimes necessary to wait several minutes after entry for the increase in amplitude to be complete. This delay may be attributed to one or both of two reasons, viz. i) recovery from

damage caused by impalement, or ii) a more efficient 'sealing in' of the microelectrode. The steady negative potential was a sum of the resting potential of the cell and the tip potential of the electrode. High resistance microelectrodes are known to suffer from high and unstable tip potentials (Kuffler and Vaughan Williams, 1953; Castillo and Katz, 1955a, 1955; Burke and Ginsborg, 1956; Adrian, 1956; Ginsborg, 1960; Fatt, 1961) particularly when they are inserted into small cells. The potential difference observed when the microelectrode was withdrawn from a cell suffered from similar disadvantages, but since the movement of the microelectrode was abrupt, the drop in the potential was easier to measure than the steady development of the potential difference on entry. The values thus obtained must nevertheless be accepted with caution (Table 1).

During the first stages of improvement of the cell, a preganglionic stimulus evoked a response which was usually bigger than the antidromic response. As the cell improved, the difference became smaller for a time, and then the antidromic response outstripped the orthodromic response, and became steadily bigger, till it was about 5 mV bigger than the orthodromic response (Fig. 5).

The average conduction velocity in the preganglionic fibres was 1.3 m/sec at 22° C. The

TABLE I

Resting potential and overshoot of antidromic action potentials.

Cell No.	Resting potential in mV.	Amplitude of spike in mV.	Peak amplitude of positive phase in mV.	Overshoot mV.
30	61	72	18	11
45	63	100	17	37
57	47	82	24	35
74	53	101	26	48
75	47	88	24	41
78	42	73	22	31
113	61	102	7	41
128	58	93	9	35
Mean: ± S.E.	54±2.8	89±5.1	18 ± 2.5	35±3.9

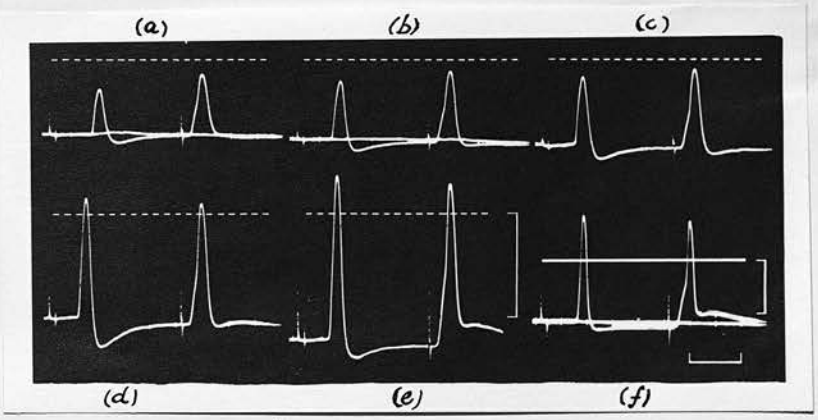
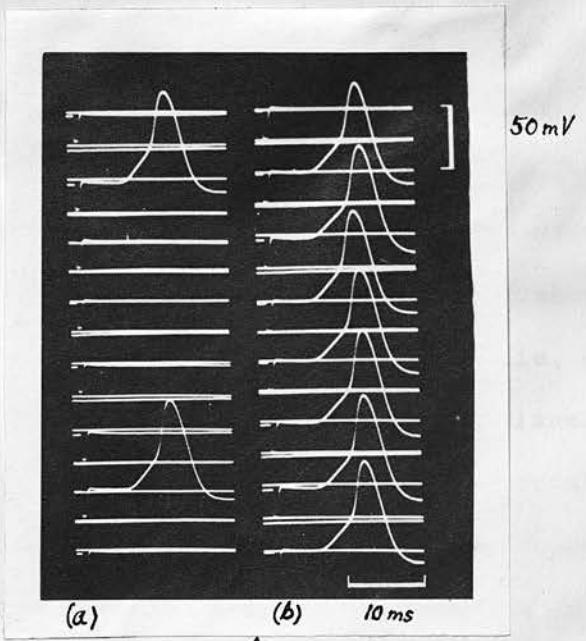


Fig. 5. Progressive increase in the responses to antidromic and orthodromic stimuli after impalement. Cell 113. Between (e) and (f) the gain was halved. At (f) the electrode was removed from the cell, giving the zero potential level. Dotted lines in (a) to (e) indicate the approximate position of the zero potential level. Voltage calibrations 50 mV, time calibration 20 ms.

RESULTS

1. Innervation

Before considering the responses to antidromic and orthodromic stimulation, it is necessary to consider the innervation of the sympathetic ganglion cell. The mammalian ganglion cell is innervated by several presynaptic axons, and stimulation of increasing intensities appears to produce graded synaptic potentials, which eventually reach the threshold for an action potential (R.M. Eccles, 1955). The situation in the frog appears to be different, the response to preganglionic stimuli (applied in the chain, see Methods) being all-or-none. For example, in the experiment illustrated in Fig. 6A, in the left-hand column, the stimulus strength used failed to produce a response in the majority of cases. On raising the strength to a supramaximal level, the responses shown on the right were obtained. No difference could be seen either in the rate of rise of the synaptic step or in the subsequent form of the action potential, and it was concluded that in the chain there was only one preganglionic fibre innervating this cell. It may be mentioned in this connexion, that plurally-innervated cells cannot be seen in the histological drawings presented by Huber (1899) and Johnson (1918).



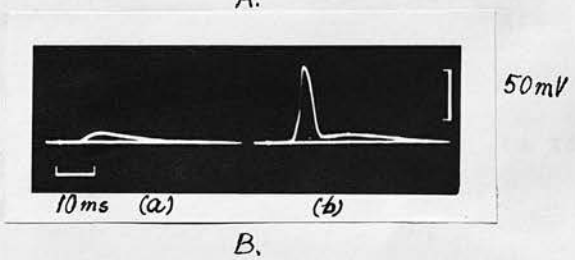


Fig. 6. Pre-synaptic innervation of two ganglion cells.

A. Cell 51. (a) Responses to a low strength stimulus, 6 out of 80 stimuli failed to excite the axon. (b) Supramaximal stimulus. (7 supramaximal stimuli) Note the identical form of the responses.

B. Cell 15. (a) and (b) as in A. Only a synaptic response resulted in (a). One or more additional axons were excited in (b).

About 70% of the cells impaled responded to stimulation from the chain. It may seem surprising that cells in the tenth ganglion, for example, apparently receive their presynaptic innervation from rami entering the chain as far distant as the seventh ganglion; however it has been shown by Bishop and O'Leary (1938) that in the thoracic ganglia, cells in a given ganglion were not in the main innervated by axons in the rami to that ganglion. Furthermore, Langley and Orbeli (1910; cf. Hutter and Lowenstein, 1955) showed that the rami of the ninth to the eleventh ganglia mainly contain post-ganglionic axons.

Only three cells, out of a total of more than two hundred cells impaled, were found to be innervated by more than a single axon (Fig. 6B).

2. Mode of action of the synaptic transmitter

Comparison of antidromic and orthodromic responses.- Responses to preganglionic and antidromic stimuli from the same cells are illustrated in Fig. 7. Both types of responses include a positive phase, but that of the orthodromic response is generally interrupted by a negative phase. There is also a synaptic step on the rising phase of the orthodromic spike. Some of the characteristics of the responses are shown in the Tables II to IV. As was pointed out by Nishi and Koketsu (1960), the orthodromic action potential is smaller both in amplitude (by about 5 mV, see Table IV) and in the maximum value of the positive phase (by about 10 mV). The origin of the positive phase will be considered in a later section.

It has been shown by Nishi and Koketsu that the antidromic action potential is closely similar to an action potential generated by direct stimulation of the same cell. The characteristics of the 'orthodromic action potential' were suggested by Nishi and Koketsu to be due to the action of the synaptic transmitter concurrent with the usual membrane changes underlying the action potential. They have shown that the transmitter at the synapse like the transmitter at the neuromuscular junction (see

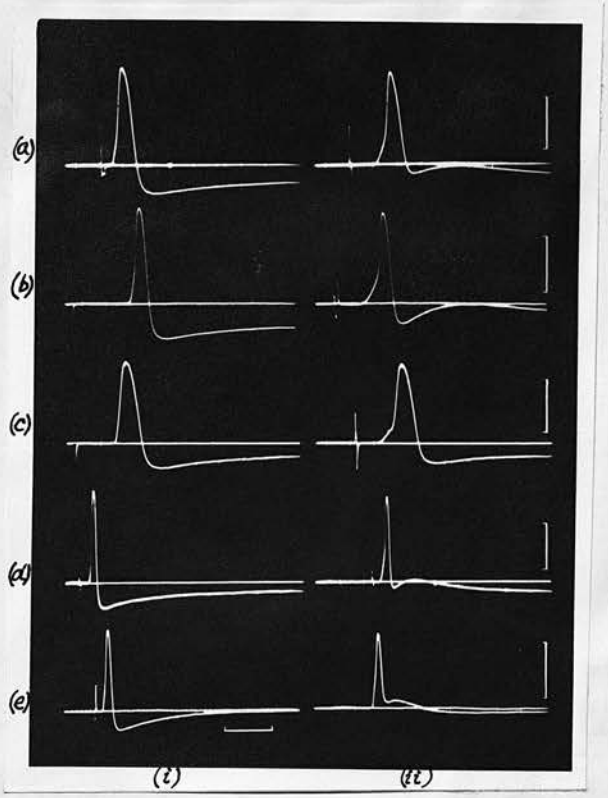


Fig. 7. Responses to antidromic and orthodromic stimulation in five cells.

(i) Antidromic response and (ii) orthodromic response.

(a) Cell 81; 50 mV, 10 ms. (b) Cell 54; 50 mV, 10 ms. (c) Cell 80; 50 mV, 10 ms.

(d) Cell 115; 50 mV, 20 ms. (e) Cell 30; 50 mV, 40 ms.

TABLE IIA

Characteristics of antidromic action potentials from 24 cells.

Amplitude of spike in mV	Peak amplitude of positive phase in mV
84 ± 1.9	23 ± 1.3

TABLE IIB

Rise-time at two different temperatures.

Temperature in °C.	Amplitude of spike in mV	Rise-time in ms.
16 - 17	82 ± 3 (7 cells)	3.4 ± 0.4
22 - 23	83 ± 5 (6 cells)	2.3 ± 0.2

(Values given are ± S.E. of mean.)

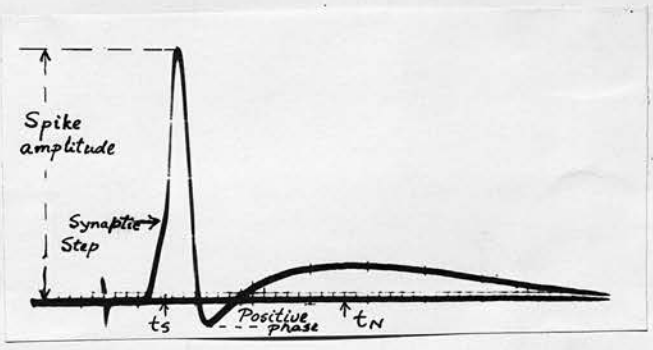


Fig. 7A. Nomenclature used in Table III for the various phases of the orthodromic response.

TABLE III

Spike	Amplitude of the		Time to synaptic step(t_S)	Time to peak negative wave(t_N)
	Synaptic step	Peak positive phase		
78±1.5	22±3.4	16±1.6	3.4±0.6 (10 cells)	21.7±1.4 (9 cells)

Characteristics of orthodromic responses from 16 cells. (Values given are ± S.E. of mean.)

TABLE IV

Comparison of Orthodromic and Antidromic responses.

Cell No.	Amplitude of the antidromic spike in mV	Amplitude of the orthodromic spike in mV	Difference in spike height in mV	Difference in positive phase in mV
8	88	88	0	4
9	76	74	2	3
12	78	74	4	10
39	94	88	6	20
53	81	73	8	2
54	83	78	5	11
71	88	77	11	3
78	79	77	2	7
81	93	87	6	13
115	91	86	5	21
Mean differences			4.8 ± 1.04	9.4 ± 2.2

(Values given are $\pm$ S.E. of mean)

Katz, 1962) produces, in effect, a relatively low resistance shunt across the membrane. The action of the transmitter would, therefore, be expected to reduce both the spike and the positive phase.

Interaction of effects of preganglionic and antidromic stimulation.- The experiments illustrated in Figs. 8 and 9 are consistent with this hypothesis (Castillo and Katz, 1954). Figure 8 illustrates experiments on three different cells. In columns (A) and (O), the responses to antidromic and orthodromic stimuli respectively are shown. The responses shown in column (A + O) were obtained by stimulating the cell preganglionically during an antidromic action potential. The preganglionic stimulus was so timed that in the absence of a pre-existing action potential in the cell, the beginning of the orthodromic response would have occurred at the points marked by the arrows. Since the cell is refractory, the preganglionic stimulus is unable to elicit an action potential; nevertheless, it produces a change in the form of the 'antidromic response'. It is evident that the resultant response closely resembles the corresponding orthodromic response.

Figure 9 illustrates an experiment in which the interaction of the effect of preganglionic stimulus with the 'antidromic action potential' has been

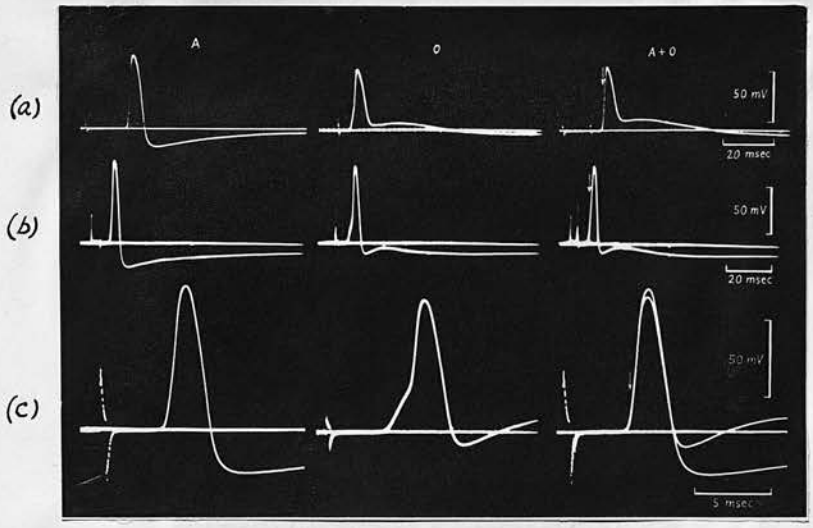


Fig. 8. Action of the transmitter on the antidromic response in three cells. (A) Antidromic response, (O) orthodromic response, (A+O) antidromic response with synaptic activity induced on the rising phase at the arrow. (a) Cell 99, (b) cell 125, and (c) cell 39. A normal antidromic response has been superimposed in cell 39 (A+O). (The point at which synaptic activity started has been calculated from the latency of the orthodromic response.)

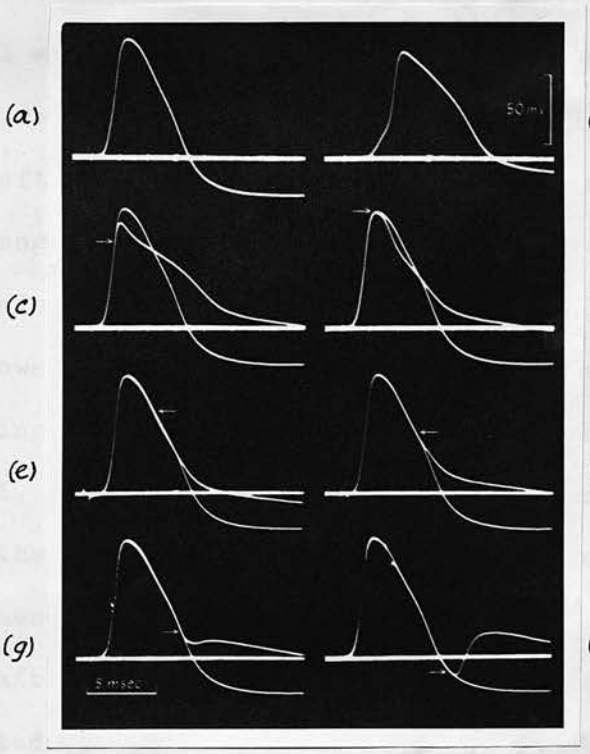


Fig. 9. Action of the transmitter on the antidromic action potential. Cell 158. (a) Antidromic response, (b) orthodromic response, (c) to (h) antidromic response during which synaptic activity has been induced (at points indicated by arrows as in Fig. 8) superimposed on a control antidromic response. The transmitter equilibrium potential lies between 60 and 40 mV above the base-line - between (e) and (f).

examined at various stages of the 'antidromic action potential'. The interval between the two stimuli was altered in small steps, and the composite action potential ~~was~~ recorded. A control antidromic response was recorded on the same trace. The top row shows on the left a normal antidromic response, and on the right a normal orthodromic response. As in Figure 8, transmitter action was induced at the points marked with arrows. When the transmitter action was initiated during the rising phase of the antidromic action potential, as in the experiments illustrated in Fig. 8, the amplitude of the spike was reduced (Fig. 9c). When it was initiated at the peak of the spike or just after it (d and e), the falling phase was accelerated (e was at a level of about 55 mV above the resting level). Induced at a level less than 40 mV above the resting base line, the transmitter caused the falling phase to be slowed (f, g and h). It may be inferred that at some potential level between 40 and 55 mV above the base line, the transmitter would have caused no change at the moment of initiation. This result is in agreement with the conclusion of Nishi and Koketsu, who found that the 'shunt' induced by the transmitter was in series with a potential difference of about -15 mV with respect to zero, which corresponds to a level of 50 mV above the base line.

Analysis on the basis of a model circuit.- The results of the experiments described by Nishi and Koketsu (1960), and those described above are simply interpreted on the basis of the model circuit shown in Fig. 10, a circuit which has been shown to apply at other synapses (cf., for example, Fatt and Katz, 1951; Eccles, 1961). The left hand branches represent the non-chemosensitive membrane and the right hand branch represents the action of the transmitter. In the left hand branches, the resting potential is represented by the battery E, the membrane resistance by R, the capacitance by C. Approximate values of R and C are given by Nishi and Koketsu as 20 MΩ and 0.5 nF respectively. The action of the transmitter may be represented by closing the switch Sw. The cell membrane is then shunted by a resistance r in series with a battery e, which represents the equilibrium potential of the transmitter. In the experiments of Nishi and Koketsu, steady currents were passed through the cell membrane, and the potential difference, V, between the outside and the inside could be varied at will. The value of e was inferred from the value of V, at which preganglionic stimulation had no effect on V. When V differed from e, preganglionic stimulation initially caused a change in the membrane potential directed

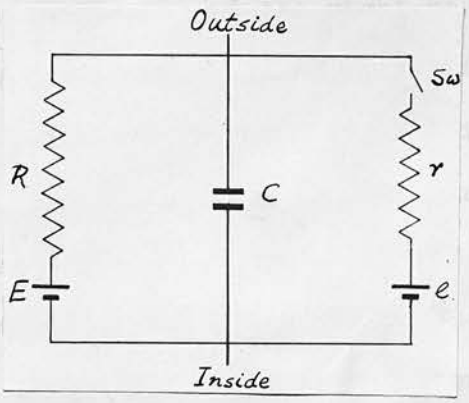


Fig. 10. Equivalent electrical circuit for transmitter action (see text). R, membrane resistance; E, membrane potential; C, cell membrane capacity; r, additional resistance interposed by the transmitter; e, transmitter equilibrium potential; SW, switch, closure of which imitates the initiation of transmitter action.

towards e and proportional to V-e.

That this model is also consistent with the results of the experiments described in the previous section, will now be shown. It will be convenient to analyse the circuit shown in Fig. 11, where, as before, the left hand branch represents the non-chemosensitive membrane and the right hand branch the chemosensitive membrane. If an antidromic action potential is generated, E and G (=1/R) will vary with time. Let E' represent the membrane potential measured from the resting base line, and G' the membrane conductance at any point during the action potential. Let V' be the recorded potential difference across the membrane.

During an antidromic action potential,

$$G' \,.(E' - V') = C \frac{dV'}{dt} \qquad (1)$$

where G' and E' are unknown functions of time and V'.

If an action potential and the transmitter action exist concurrently (as during the orthodromic action potential), and the resulting potential difference across the membrane is V"

$$G''. (E''-V'') + g.(e - V'') = C \frac{dV''}{dt} \qquad (2)$$

At the instant of induction of transmitter action on the antidromic action potential, V' = V", G" = G' and E" = E', therefore

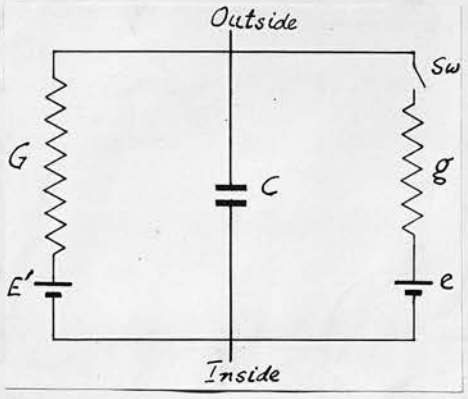


Fig. 11. Equivalent electrical circuit for action of transmitter on the antidromic action potential (see text). Symbols as in Fig. 10, but E′ and e are measured with respect to the resting base-line and not with respect to zero potential level.

24.

$$\frac{dV''}{dt} - \frac{dV'}{dt} = \frac{g}{c}. (e - V') \qquad (3)$$

For values of V' greater than e, the differences in the rates of change of potential will have negative values, i.e. the rising phase of the antidromic action potential will be retarded by the concurrent action of the transmitter and the falling phase will be accelerated. For values of e greater than V', the difference in the rate of change of the potential will be positive, i.e. the rate of decay of the antidromic action potential for example, will be slowed by the co-existence of the transmitter action.

3. The after-hyperpolarization

The records which have already been reproduced show, in agreement with Nishi and Koketsu (1960), that the action potential obtained in response both to preganglionic and antidromic stimulation consists of a spike followed by a positive phase. The origin of the positive phase will be considered in this section. Since the positive phase of the orthodromic action potential is modified by the action of the transmitter, attention will mainly be paid to the positive phase of the antidromic action potential. Its mean maximum amplitude was 23 mV ± 1.3 mV (S.E. of the mean in 24 cells), and it usually decayed to half this value in 20 to 30 ms, but in some cells much slower rates of decay were seen (Fig. 12).

Effect of changes in the external ionic concentration on the action potential.- Figure 13 illustrates the effect of increasing the external potassium concentration on two cells. Both the resting potential and the positive phase are reduced by an increase in the potassium concentration. The effect on the positive phase was very much more marked than that on the resting potential. In seven cells, a change in the external concentration of potassium from 2 mM to 4.7 mM gave rise to a change of 9 mV in the level of the peak of the positive

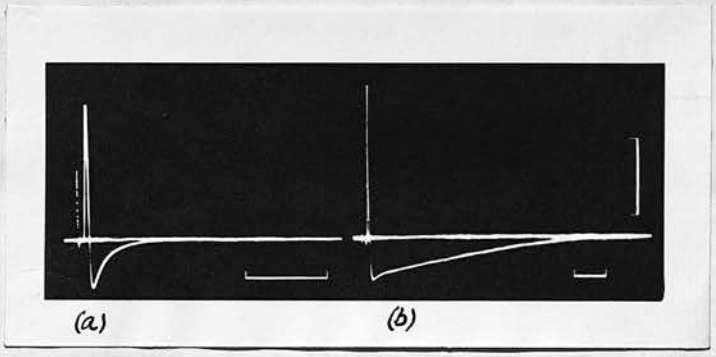


Fig. 12. Antidromic responses from two cells showing different time courses of the positive phase . (a) Cell 39 and (b) cell 74. Voltage calibration 50 mV, time calibrations 50 ms.

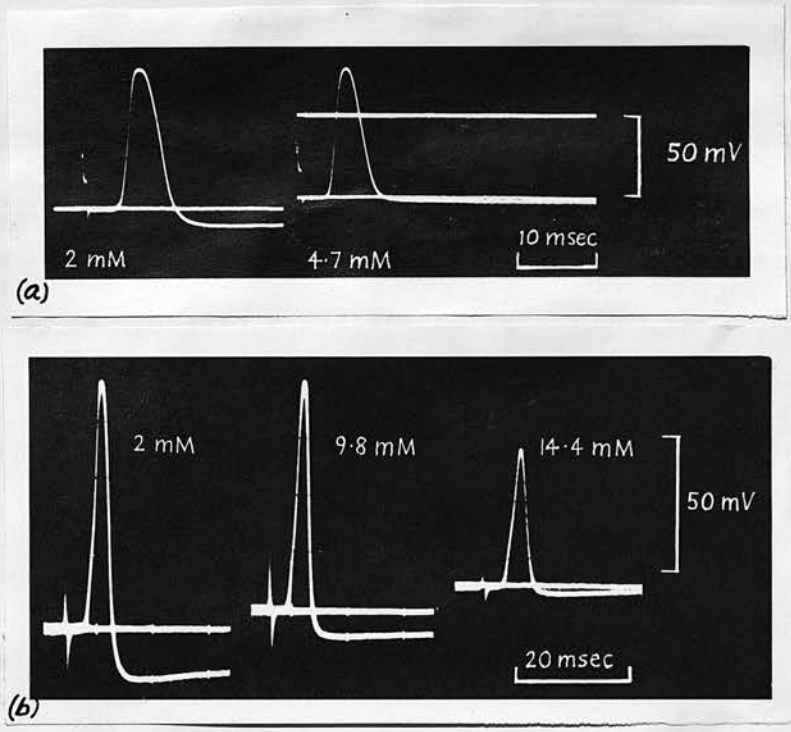


Fig. 13. Effect of increase in external K^+ concentration on the antidromic action potential of two cells. K^+ concentrations shown in figure. (a) Cell 128, chloride-Ringer's fluid. Zero potential level obtained on removal of electrode from the cell. (b) Cell 137, methylsulphate-Ringer's fluid.

phase and of 3mV in the resting potential (see Table V).

For small changes in potassium concentration, and hence in resting potentials, there was little or no change in the level of the peak of the spike. However, for depolarizations of about 10 mV or more there was a marked reduction in this level.

On changing the potassium concentration, the changes in resting potential and the positive phase occurred rapidly (Fig. 14).

In several experiments, the effect of changing the concentration of potassium in Ringer's solution where the chloride had been replaced by methylsulphate was observed. Similar changes in the positive phase and the resting potential occurred as in Cl^--Ringer solution. Qualitatively, these results are best explained on the assumption, that as in cephalopod axons, the resting potential in sympathetic ganglion cells is smaller in absolute value than the potassium equilibrium potential, and that the decaying phase of the action potential is associated with an increase in the membrane permeability to potassium ions (Hodgkin and Katz, 1949; Hodgkin and Keynes, 1955; Hodgkin and Huxley, 1952c).

Effects of repetitive stimuli.- Figure 15 shows the effects of trains of stimuli at about 12 per

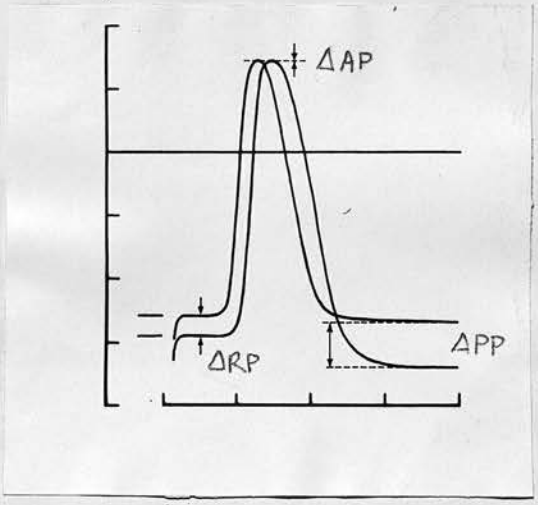


Fig. 14A. Nomenclature for Table V (Cell 128).

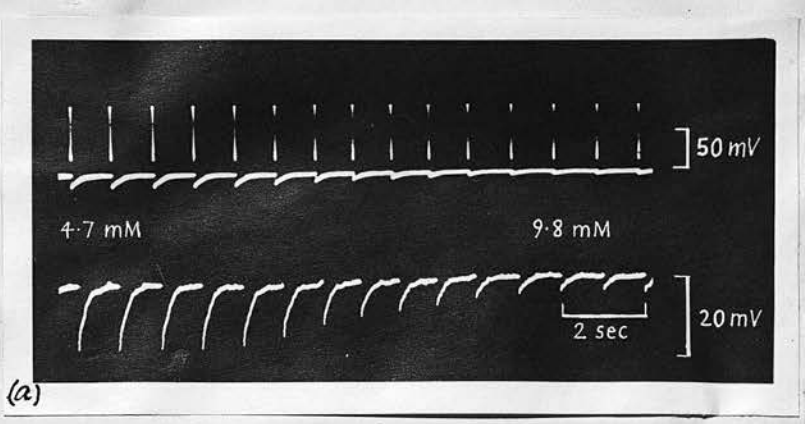
TABLE V

Effect of altering the external potassium concentration on the antidromic action potentials.

Cell No.	2 mM K+			4.7 mM K+			7.4 mM K+		
	AP in mV	RP in mV	PP in mV	ΔAP in mV	ΔRP in mV	ΔPP in mV	ΔAP in mV	ΔRP in mV	ΔPP in mV
125	99	48	29	8	3	13	-	-	-
126	71	-	19	-	-	-	6	7	19
128	86	-	11	0	4	12	-	-	-
130	69	-	13	3	9	14	-	-	-
135	71	-	9	0	3	11	-	-	-
136	73	-	3	5	6	9	-	-	-
137	95	56	19	0	0	0	0	7	15
159	82	-	12	-	-	-	15	6	14
166	71	-	28	3	2	5	20	6	19
Mean				3	4	9	10	6.5	17

AP - Amplitude of the 'spike' measured from the resting base-line; RP - Resting potential measured from the zero level; PP - Peak amplitude of the 'positive phase' measured from the resting base-line.

Cells 130 to 137 were in methylsulphate Ringer's fluid.



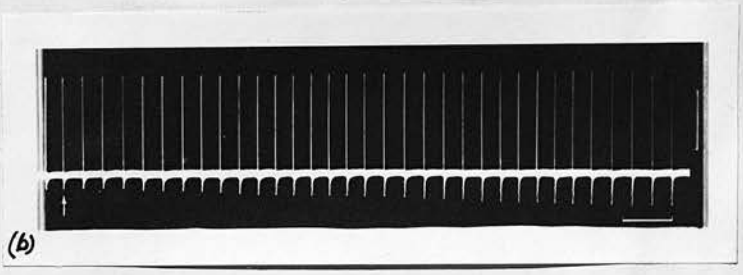


Fig. 14. Effect of altering the external K^+ concentration on the antidromic responses in two cells.

(a) Cell 137, methylsulphate-Ringer's fluid. The responses were recorded at two different gains. The top trace shows the full response and shows no change in the spike height. The bottom trace (at higher gain) shows the change in the resting level and in the peak amplitude of the positive phase.

K^+ added to bath 10 sec. before the first action potential.

(b) Cell 173, chloride-Ringer's fluid. K^+-free solution run into the bath, starting at the arrow and continuing throughout the period shown.

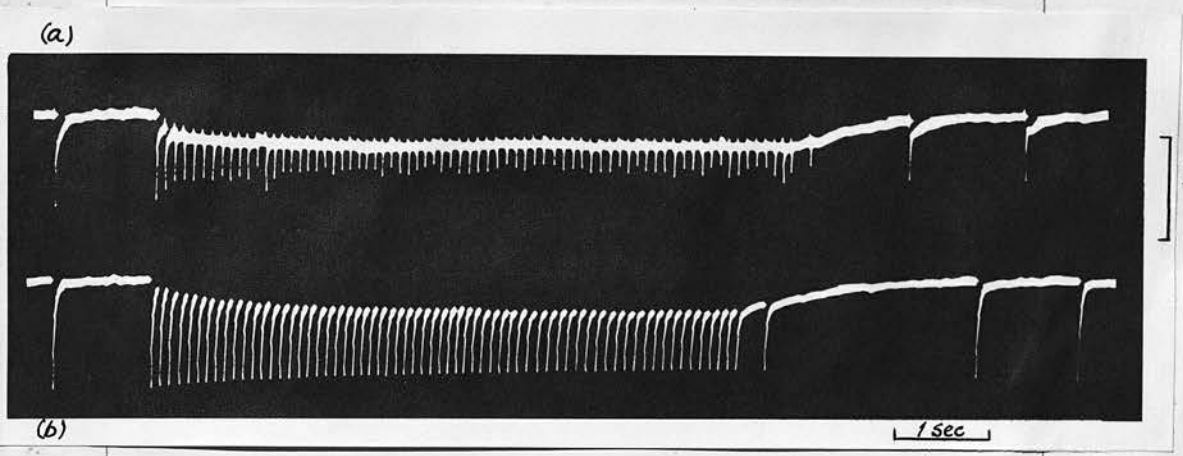


Fig. 15. Effect of repetitive stimuli (at about 12/sec) on the 'positive phases' of (a) the orthodromic responses and (b) the antidromic responses. Cell 183. Only the 'positive phases' are seen in the recording conditions used (at high gain and on a slow time-base). Note the decrease in the peak amplitude of the positive phases and the increase in the absolute value of the resting level.

second on the positive phases of both the orthodromic and the antidromic responses. It will be observed that successive positive phases are reduced in amplitude. This effect is consistent with the idea that the positive phase represents an increase in potassium permeability of the cell (Frankenhaeuser and Hodgkin, 1956), since it is most easily explained by assuming a local accumulation of potassium around the cell membrane during the train of impulses.

An additional effect observed is that during the train of impulses, the absolute value of the potential level between impulses slowly increases, and at the end of the train the resting potential returns to its usual value only slowly. This effect might be due to a gradual prolongation of the increase in potassium permeability, or alternatively, to a slow decrease in the sodium permeability during the train.

Effects of changing K^+ on orthodromic responses.- Figure 16 illustrates the effect of changing the concentration of potassium on the antidromic and the orthodromic responses of the same cell. In addition to the reduction in the positive phase, it is seen that the orthodromic response fails at a potassium concentration of about 10 mM. This fact was confirmed in many other cells. The failure is

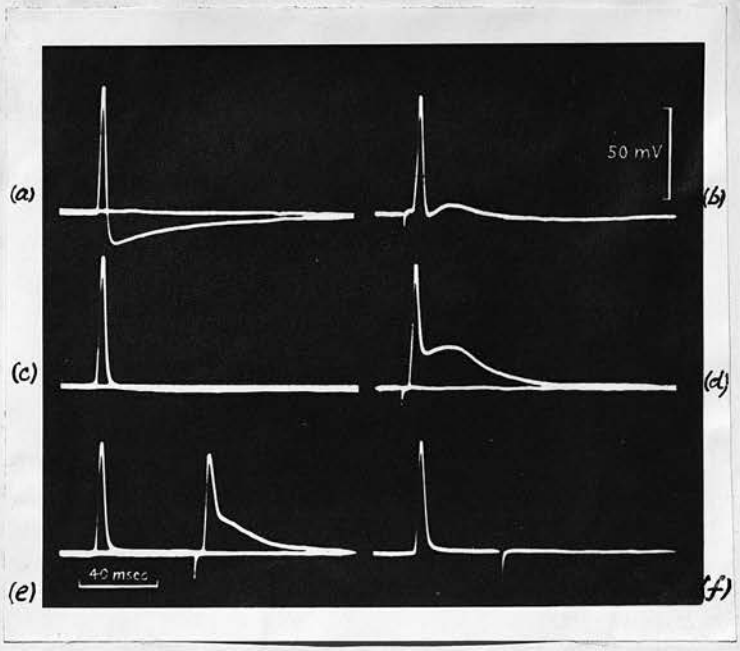


Fig. 16. Effect of increase in external K^+ concentration on the antidromic and orthodromic responses from the same cell. Cell 135, methylsulphate-Ringer's fluid.
(a) Antidromic response and (b) orthodromic response in 2 mM K^+. (c) Antidromic and (d) orthodromic response in 4.6 mM K^+.
(e) 7.1 mM K^+, antidromic and orthodromic responses on the same trace. (f) 9.6 mM K^+, note the orthodromic response is blocked.

evidently presynaptic, since an antidromic response is obtained at that concentration. Furthermore, at a higher concentration of potassium at which the antidromic stimulus fails to generate a full response, spontaneous synaptic action (see Part II) is capable of generating an action potential (Fig. 17). It must therefore be presumed that the cell is still excitable and that 'depolarization block' has occurred in the conduction pathway to the cell. The small size of the antidromic response (in Fig. 17b) may be explained by supposing that the action potential generated in the post-ganglionic axon is shunted by the depolarized membrane of the cell body to such an extent that it fails to reach the threshold to fire the cell. The spontaneous synaptic activity is itself however generated across a relatively low resistance and is therefore less affected by the low resistance of the cell body.

It should be pointed out that the antidromic response is normally produced in two stages - the first stage being the spike in the axon immediately adjacent to the cell body (see Nishi and Koketsu, 1960; cf. Coombs, Curtis and Eccles, 1957). This spike is normally shunted by the relatively low resistance of the cell body, because of the surface expansion, but it is nevertheless large enough to reach the threshold for an action potential in the cell body membrane.

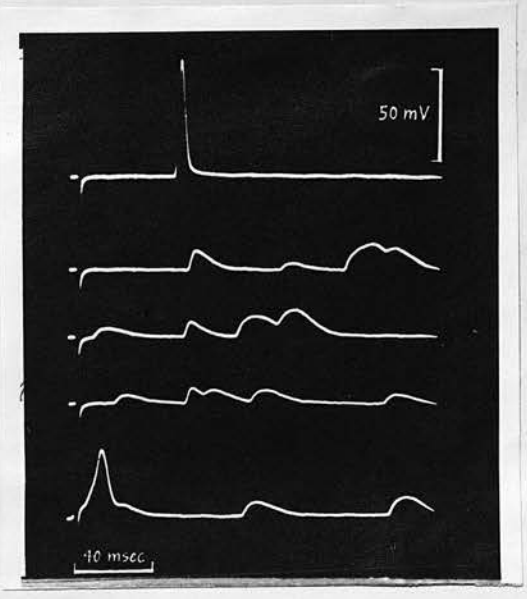


Fig. 17. Effect of high external K^+ concentrations on the antidromic responses. Cell 135, methylsulphate-Ringer's fluid. (a) 9.6 mM K^+, (b) 11.8 mM K^+, note miniature synaptic potentials and small antidromic response on the top three traces; in the bottom trace a spontaneous action potential has blocked the antidromic response.

4. Action of blocking agents

On the basis of experiments performed on the superior cervical ganglion of cats, Paton and Perry (1953) classified the drugs blocking transmission in autonomic ganglia into two classes, viz. a) drugs that blocked in the absence of depolarization, and b) drugs that caused an initial depolarization and caused a block of transmission by depolarization. Drugs in the first category include (+)-tubocurarine, pentamethonium, hexamethonium (cf. Pascoe, 1956), eserine in large doses. In the second category were tetramethylammonium, nicotine and acetylcholine. Nicotine had a dual action as confirmed by Lundberg and Thesleff (1953), it produced a depolarization and block, but the block persisted after the decay of the depolarization.

Non-depolarizing blocking agents.- Figure 18 illustrates a typical sequence of block of response to orthodromic stimulation on the addition of a non-depolarizing blocking drug (in this case mecamylamine to 2×10^{-4} M) to the bath. The most obvious initial effect is the reduction of the negative phase following the spike, and the associated increase in the positive phase. In addition the rate of rise of the synaptic step is progressively slowed;

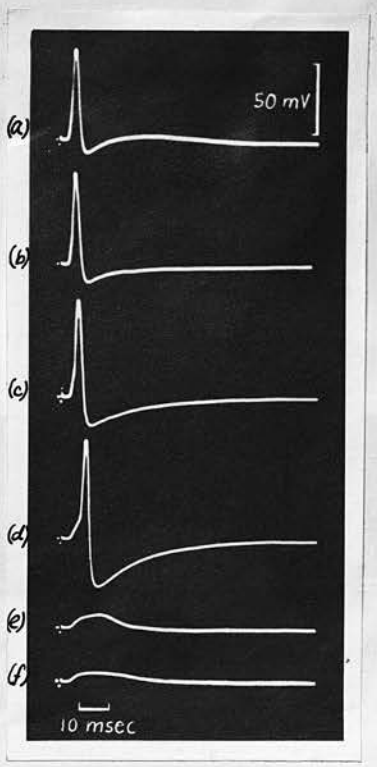


Fig. 18. Progressive block of transmission by mecamylamine. Cell 43. (a) Normal response. Note progressive increase in the 'positive phase' in (b) to (d). There may be a component of 'local response' in (e). The mecamylamine was added to the bath to make the final concentration 2×10^{-4} M. Records were then taken every few seconds. The same type of procedure was used in the experiments illustrated in Figs. 19 and 20.

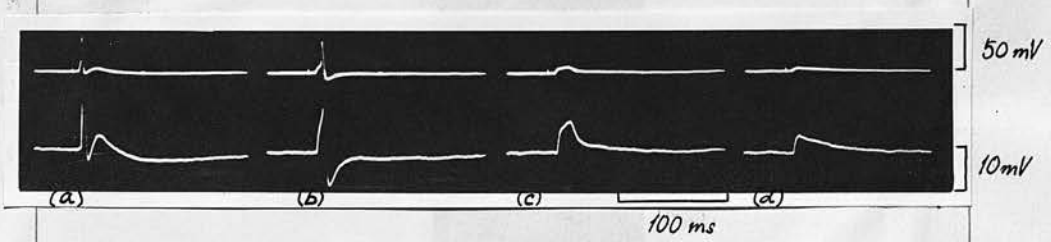


Fig. 19. Progressive block of transmission by eserine. Cell 174. The responses have been recorded at two different gains (the bottom trace at five times the gain of the top). Normal responses at the extreme left. Note component of local response in (c). Note also there was no change in the absolute value of the resting level as indicated by the absence of any change in the distance between the top and the bottom traces. Final concentration of eserine was about 3.5×10^{-4} M.

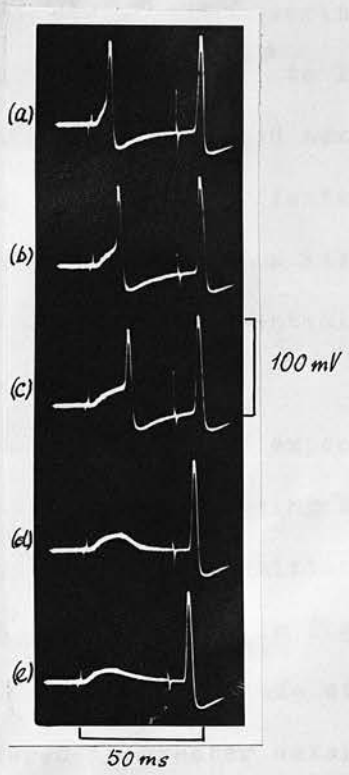


Fig. 20. Progressive block of transmission by hexamethonium. Cell 182. Each trace shows orthodromic response followed by antidromic response. (a) Normal responses. Final concentration of hexamethonium was about 7×10^{-4} M.

finally, the spike is no longer generated (there may be a component of local response in Fig. 18e) and only a synaptic potential is observed. (i.e. a sub-maximal increase in Na^+-permeability) Similar records were obtained with (+)-tubocurarine (5 to 20 X 10^{-6} M), high concentrations (10^{-5} to 10^{-4} M) of eserine and neostigmine (Fig. 19), and hexamethonium (about 7 X 10^{-4} M) (Fig. 20). The effects were rapid in onset and rapidly reversed. As is illustrated in Fig. 19, no change in resting potential or the antidromic response was observed.

These results are what would be expected on the assumption that the non-depolarizing blocking agents reduce the intensity of transmitter action. In effect, in the model illustrated in Fig. 11, the value of g has been reduced. The mode of action of curare will be considered in greater detail in Part III.

Trains of rapid preganglionic stimuli in the curarized ganglion did not show the late negative wave reported in external records of reptilian and mammalian ganglia (Laporte and Lorente de Nó, 1950; R.M. Eccles, 1952b) (Fig. 21A).

Depolarizing blocking agents.- Figure 21 illustrates the antidromic and the orthodromic responses of a cell after the addition of nicotine (10^{-4} M) to the bath. The cell was depolarized by about 9 mV and the spike heights of both the orthodromic

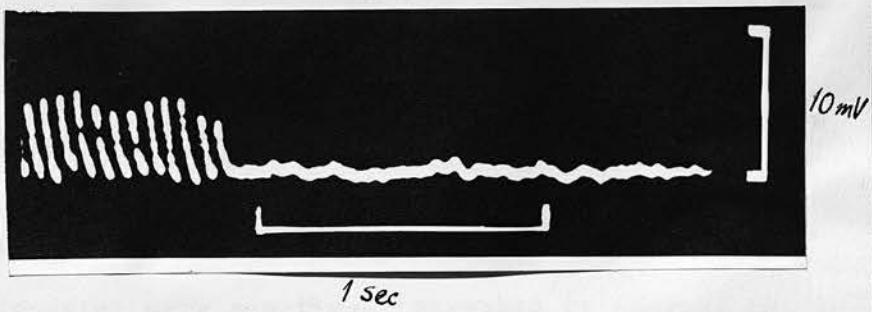


Fig. 21A. Effect of a train of preganglionic stimuli in a curarized ganglion. Cell 33, 5×10^{-5} M tubocurarine. The record shows the end of a 4-second period of stimulation at about 18/sec.

and the antidromic responses were reduced (cf. action of potassium, section 3). The orthodromic spike was eventually blocked to uncover a synaptic potential. The depolarization of the cell however passed off, and the antidromic spike was restored almost to its original height. But the orthodromic spike remained blocked. With low divided doses of tetramethylammonium, block was seen to occur without depolarization.

Anticholinesterases.- At concentrations below the blocking concentration, the duration and amplitude of the negative wave of the orthodromic action potential were sometimes increased by eserine or neostigmine (Fig. 22). Occasionally the negative wave was large enough to initiate a second spike. No effect however was seen on the rate of rise of the synaptic potential.

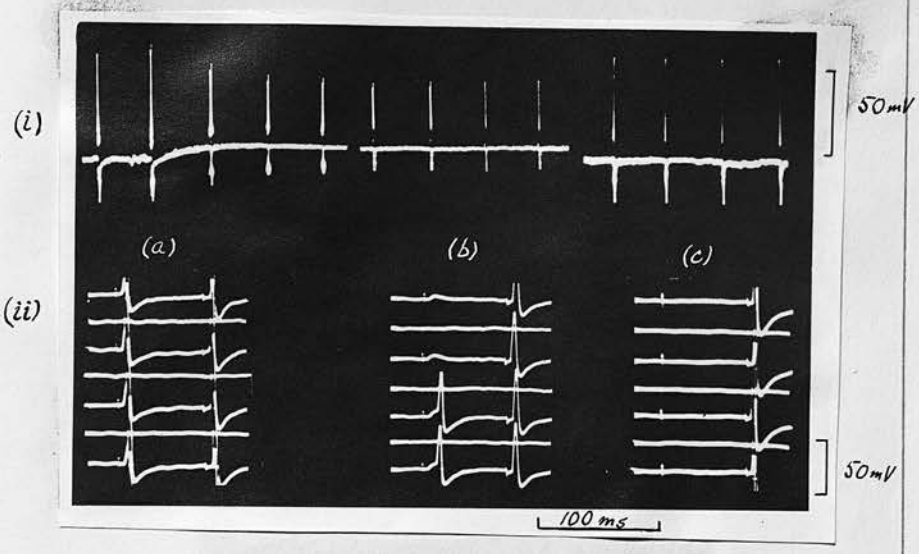


Fig. 21. Illustrates the action of nicotine on the orthodromic and antidromic responses of a ganglion cell. The responses have been recorded simultaneously at two different gains and time-bases (see Methods). Three groups of corresponding records from a continuous series are illustrated in (a), (b) and (c). The groups are arranged from left to right. In (ii), succeeding traces in each group have been recorded immediately above the preceding trace, the spikes of the action potentials overlapping slightly. The interval between each pair of responses (orthodromic, followed by antidromic) was 4 sec. The gap between (a) and (b) represents an interval of 20 sec., and that between (b) and (c) 5 min. Nicotine was added to the bath, to give a final concentration of 10^{-4} M, immediately before the first pair of responses.
Note in (a) the depolarization caused (about 9 mV) and the reduction in the amplitudes of both types of responses. In (b) the orthodromic response was blocked uncovering a synaptic potential. The depolarization has partly decayed in (c), the antidromic response has almost returned to its original amplitude, but the orthodromic response is still blocked.

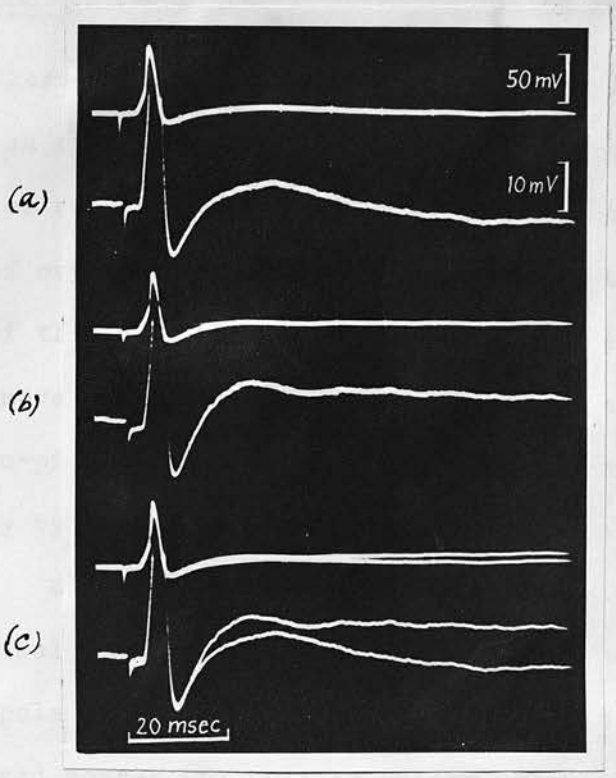


Fig. 22A. Prolongation of duration of synaptic activity by neostigmine. Cell 175. The bottom trace in each group is recorded at five times the gain of the top trace. (a) Normal response. (b) Response after addition of neostigmine to raise the concentration to about 10^{-4} M. The two responses (a) and (b) have been superimposed photographically in (c). Note the distance between the bottom and the top traces is constant indicating neostigmine did not cause a depolarization of the cell. Note also the rate of rise of the synaptic step was not affected.

5. Iontophoretic application of acetylcholine

A micro-pipette containing acetylcholine was mounted on the micro-manipulator and visually manoeuvred as near as possible to the cell impaled. Pulses of outward current were then applied to the inside of the micro-pipette, causing acetylcholine ions to move out with each pulse. Diffusion out of the micro-pipette between pulses was prevented when necessary by applying a steady negative potential to the tip. When the micro-pipette was sufficiently near the cell, each pulse of acetylcholine caused a small depolarization. The micro-pipette was carefully moved nearer the cell to obtain the maximum effect of the drug. The time to the peak of the depolarization from the moment of release represented a measure of the time required for acetylcholine to diffuse to the chemosensitive areas of the cell. The nearer the micro-pipette was to the cell, the less acetylcholine would be needed to produce a given depolarization, and the faster the rate of rise of the depolarization.

The current necessary to produce the depolarization was measured as already described (see Methods), by measuring the potential drop across a known resistance. In practice, the potential change was displayed on the second beam of the oscilloscope and

photographed at the same time as the induced depolarization of the cell. Figure 22 shows some of the records obtained. If it is assumed that all the current was carried by acetylcholine ions, then each pulse was estimated to contain approximately 1×10^{-12} moles and represented a transfer of charge equivalent to 8×10^{-8} coulombs.

The fastest potential change observed was a depolarization of 12 mV, the peak occurring at 200 ms from the beginning of the pulse. If it is assumed that the time course of the depolarization reflects the concentration of acetylcholine at the subsynaptic membrane, and that this concentration builds up in a way governed by simple diffusion from a point source, it may be calculated that the distance of the pipette was given by

$$r^2 = 6kt = 6 \times 8 \times 10^{-6} \times 200 \times 10^{-3} \text{ cm}^2$$

where r is the distance of the pipette, k is the diffusion coefficient for acetylcholine and t the time to the peak of the depolarization. From the above, $r = 30\ \mu$.

If the acetylcholine had been released from a

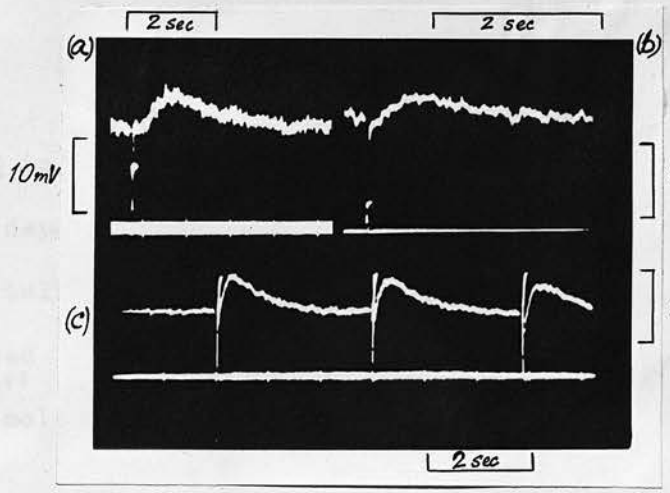


Fig. 22. Responses to iontophoretic application of acetylcholine from three cells. The lower trace of each record shows the pulse (duration 50 ms) ejecting acetylcholine monitored across a resistance (5 KΩ), and represents 8×10^{-8} coulombs in each trace. (a) Cell 8, (b) cell 1 and (c) cell 6. In (c), responses to three pulses are shown, the frequency being 1/3 sec.

point nearer the subsynaptic membrane, say 1 μ away, which is likely to be in excess of the distance between the nerve terminals and the membrane, then the amount required to produce a similar depolarization may be calculated from the relationship

$$\frac{Q}{v} \propto r^3$$

where Q is the amount of the drug and v the amplitude of the depolarization produced. The amount of acetylcholine needed to produce the same effect when liberated 1 μ away is, therefore, not more than 3×10^{-17} moles.

DISCUSSION

It will be convenient to discuss the results which have been described under two separate headings, namely

(i) The identity of the synaptic transmitter and the action of the blocking agents,

(ii) The mode of action of the transmitter.

Identity of the synaptic transmitter and action of the blocking agents

The results described in sections 4 and 5 are consistent with the idea that acetylcholine is the synaptic transmitter in the sympathetic ganglia of the frog. Ganglion cells were depolarized by micro-application of a relatively small quantity of acetylcholine (10^{-12} moles). Furthermore, plausible calculations based on the observed sensitivity of the cells to acetylcholine suggests that a very much smaller quantity would have been required to initiate an action potential had it been possible to achieve better localization of the micro-pipette. In fact it was shown that a depolarization of about 12 mV would have been produced by the application of 3×10^{-17} moles of acetylcholine 1 μ away from the subsynaptic membrane, and presumably the application of 6×10^{-17} moles would have achieved a depolarization

sufficient to fire the cell. This amount appears to be of the same order of magnitude as that released per preganglionic volley per cell in the mammal (10^{-17} moles in the cat as estimated by Eccles (1953) from figures quoted by Feldberg and Vartiainen (1935), Perry (1953) and Emmelin and MacIntosh (cf. 1956)).

A second line of evidence comes from the action of anticholinesterases. In appropriate concentrations, the amplitude and the duration of the negative wave of the orthodromic action potential were increased. The negative wave has been shown (section 2) to be due to the residual action of the transmitter, and it therefore seems reasonable to suppose that its enhancement by anticholinesterases indicated that the transmitter is acetylcholine. It is not surprising that no intensification of the initial phase of transmitter action was observed under the influence of anti-acetylcholinesterases (the rate of rise of the synaptic step was not increased). In the first place, there is no evidence that the cholinesterase in the frog sympathetic ganglion exists in high concentration in the subsynaptic membrane (Giacobini, 1956). Cholinesterases may therefore be presumed to play a minor role in the removal of acetylcholine and the inactivation of cholinesterases would be expected to play only a minor role in intensifying

the action of acetylcholine. Secondly, anticholinesterases also block the frog's sympathetic ganglion as they do the mammalian sympathetic ganglion, and a certain degree of reduction in sensitivity to acetylcholine may be occurring in parallel with the increase in concentration induced by inactivating cholinesterase.

A third line of evidence for acetylcholine as the transmitter is indicated by the action of blocking agents. In this connexion, the first point of interest is that agents which cause blockade at the mammalian ganglion do so also at the frog's ganglion; secondly, the classification of Paton and Perry (1953) of depolarizing and non-depolarizing drugs holds also for the frog. It seems unlikely that there should exist a fortuitous similarity between the behaviour at non-cholinergic and cholinergic receptors.

With regard to the mode of action of the non-depolarizing blocking drugs, it may be tentatively assumed that they act post-synaptically by combining with the specific receptors for acetylcholine. A direct action on the cell membrane in any other way would seem to be ruled out by the fact that the blocking agents tested had no effect on the amplitude or form of the antidromic action potential. It might however be argued that a presynaptic action was involved. The only direct proof that this was not

the case would involve collection of the transmitter from a perfused frog's ganglion, which has not been attempted. In the case of tubocurarine however, another approach has been adopted and will be described in Part III. At this stage it may be pointed out that the results which will be described suggest that the action of tubocurarine is post-synaptic. No information can be given on the effects described by Laporte and Lorente de Nó (1950) and R.M. Eccles (1952b, ~~1961~~). In the external records of curarized reptilian (turtle) and mammalian (rabbit) sympathetic ganglia, trains of preganglionic stimulation produced a late negative wave lasting for seconds. No such effect was observed in intracellular records in the present investigation. It is not known however whether such potentials are seen externally in the frog.

Although it is true that the classification of blocking drugs into depolarizing and non-depolarizing drugs (Paton and Perry, 1953) holds for the frog, it is also evident from the results described previously that the depolarizing blocking drugs do not in fact necessarily block by depolarization. Thus in Fig. 21, it is evident that block occurs after peak depolarization has occurred, transmission is <u>not</u> restored after the membrane potential is restored and the antidromic response is not blocked at any stage.

The amplitude of the antidromic action potential is greatly reduced; this appears to be the result of depolarization, as such, and not the result of the action of the drug, since a depolarization of similar magnitude induced by an increase in external potassium concentration also leads to a similar reduction in the amplitude of the response to antidromic stimulation (Fig.13).

The mode of action of the transmitter

The nature of the action on the cell membrane produced by the synaptic transmitter does not require much comment. The results described are largely confirmatory and agree with those of Nishi and Koketsu (1960) in indicating that the action of the transmitter, just as at the neuromuscular junction (Fatt and Katz, 1951), is to 'shunt' or 'short circuit' the membrane with an additional conducting pathway. This pathway is equivalent to a resistance in series with the 'transmitter equilibrium potential' whose absolute value (about -15 mV) is considerably less than that of the resting potential. The cell membrane as a whole is therefore depolarized and the depolarization sets off an action potential. The action of the transmitter is prolonged and its continued tendency to hold the membrane towards its equilibrium potential reduces the 'overshoot' and subsequent 'undershoot' (or the positive phase) of the orthodromic action potential as compared to those of the antidromic action potential. The prolonged action of the transmitter is in line with the absence of strategically placed cholinesterase in the post-synaptic membrane; it will be recalled that after the inactivation of cholinesterase, the duration of transmitter action at the neuromuscular junction

is also of relatively long duration (Fatt and Katz, 1951). It is also worth mentioning that the prolonged action of the transmitter in the frog is not related to dispersion of conduction along the presynaptic pathway, since in the experiments described, only a single presynaptic axon was involved (section 1).

The implications of the value obtained for the transmitter equilibrium potential have been discussed in detail by Castillo and Katz (1954) at the neuromuscular junction. It is consistent with a generalized increase in permeability to all the small ions on both sides of the membrane. At the neuromuscular junction however the evidence (Takeuchi and Takeuchi, 1960) points to the absence of an increase in permeability to chloride ions. More refined investigations will be required at the ganglion to distinguish between the different possibilities. What is certain is that the increase in permeability is not confined to sodium ions alone, as during the rising phase of the action potential.

The positive phase of the action potential does not appear to be related to the action of the transmitter - a conclusion reached as early as 1936 (Eccles) - since a positive phase occurs also in the antidromic action potential. The behaviour of the positive phase to changes in ionic concentrations suggests that like that of the squid axon, it is due to

'delayed rectification', i.e. the delayed and prolonged increase in permeability to potassium ions, following the rising phase of the action potential. An analysis of the ionic factors controlling the resting potential and of the membrane changes underlying the action potential has not been attempted. It would be made difficult by the absence of a suitable substitute for sodium ions and by the uncertain effects of 'leakage' around the micro-electrode.

PART II

INTRODUCTION

It is known that at the vertebrate neuromuscular junction, acetylcholine is always released in the form of multimolecular 'packets' or quanta (Fatt and Katz, 1952; Katz, 1962). When the motor nerve is stimulated, several hundreds of such quanta are released simultaneously, and they give rise to an end-plate potential. In the absence of stimulation, individual quanta are released spontaneously at random intervals, and each of these gives rise to a 'miniature end-plate potential'.

The fact that spontaneous synaptic potentials appear to occur in the sympathetic ganglion cells of the frog (Nishi and Koketsu, 1960) suggests that the mechanism of release of acetylcholine might also be in the form of quanta at that site. The characteristics of the spontaneous synaptic potentials will be considered in this part and the evoked release in Part III.

METHODS

The recording arrangements and the general methods of impalement have already been described (Part I). In most of the experiments described, the intracellular potentials were displayed simultaneously on two oscilloscopes connected in parallel. One of the oscilloscopes yielded the usual form of records at fixed sweep speeds on separate frames of the recording film. The other oscilloscope was arranged so that the trace moved freely in a vertical plane. This yielded continuous records of all events occurring in the cell on a minimum of film. The amplifiers connected to the oscilloscopes were set at high gain.

One major difficulty of recording small potential changes at high gain was the random fluctuations of the baseline. This 'noise' was sometimes greater than 0.5 to 1 mV; this was more than that accounted for by the resistance of the microelectrode (about 150 μV r.m.s. for a 30 MΩ microelectrode at 22° C for a frequency response of the system 0 to 30 Kcs, or about 400 μV peak-to-peak). Furthermore, the noise was often reduced on withdrawal of the microelectrode from the cell, without a change in microelectrode resistance. Concentrations of (+)-tubocurarine that blocked synaptic transmission in the cell did not reduce the 'noise'; presumably the 'noise' was not

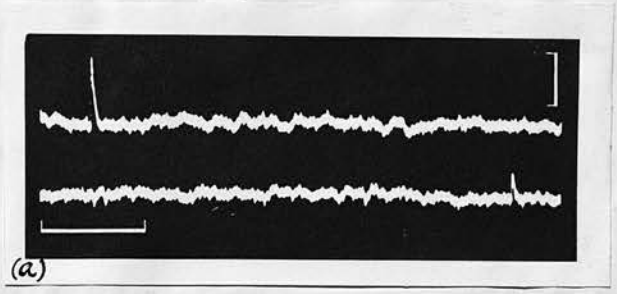
due to acetylcholine leaking from the presynaptic terminals. One procedure that almost invariably reduced the amplitude of the 'noise' considerably was raising the concentration of potassium in the bath (see Fig.29). A possible explanation of this phenomenon is that the 'noise' was caused by fluctuations in the 'membrane seal' around the tip of the micro-electrode. It can be shown that if such was the case, then procedures that reduced the membrane resistance would cut down the 'noise' appreciably. Although no experimental evidence is available that raising the external concentration of potassium reduces the resistance of the ganglion cell, it would be expected to do so on theoretical grounds (e.g. Hodgkin and Katz, 1949).

RESULTS

1. Spontaneous synaptic potentials

Intracellular records from ganglion cells showed, in about a third of the cells impaled, small sub-threshold depolarizations occurring spontaneously (i.e. in the absence of overt stimulation) (Fig. 23), which could be distinguished from the baseline 'noise'. The characteristics of the depolarizations from a number of cells is given in Table VI. Several results, to be considered below in greater detail, suggest that these spontaneous depolarizations are in fact 'miniature synaptic potentials' analogous to the 'miniature end-plate potentials' seen at the neuro-muscular junction and are due to quanta of acetyl-choline released from the nerve terminals. They were seen in ganglia poisoned with magnesium, and their time course resembled that of the evoked synaptic potentials in such cells. Their amplitude was reduced by (+)-tubocurarine in concentrations that blocked synaptic transmission (Fig. 24). That they were not due to nervous excitation was indicated by the fact that they occurred in cells, the preganglionic axons to which had been made inexcitable by increasing the external potassium concentration (Fig. 25) (cf.Fig.16).

Frequency.- The frequency of occurrence of the



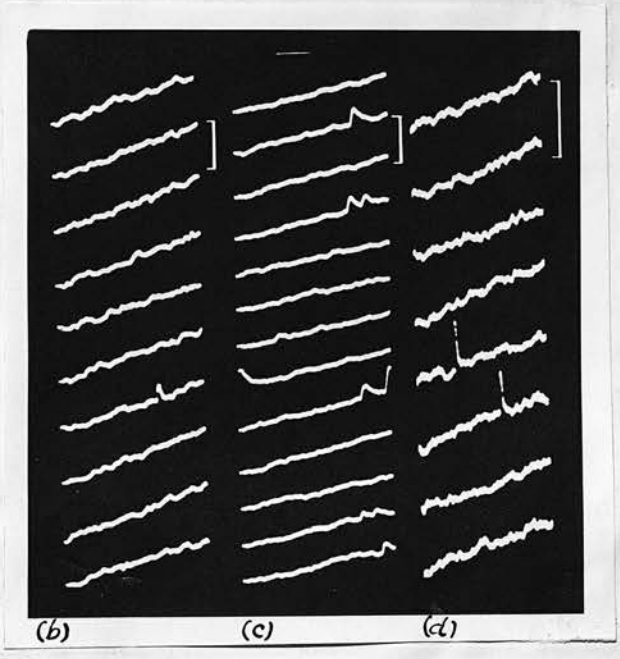


Fig. 23. Miniature synaptic potentials from four cells. (a) Cell 49, 5 mV, 1 sec. (b) Cell 83, 10 mV, trace length 1250 ms. (c) Cell 113, 10 mV, trace length 350 ms. (d) Cell 68, 5 mV, trace length 1 sec.

TABLE VI

Some characteristics of miniature synaptic potentials

Cell No.	Amplitude of evoked response in mV	No. of miniature synaptic potentials recorded	Frequency	Amplitude of miniature synaptic potentials in mV		
				Mean	Range	S.D.
1	65(A)	93	8/sec.	1.7	0.5-6.4	0.8
113	102(A)	65	1.5/sec.	2.2	1.0-6.0	0.93
81	90(A)	35	1/sec.	3.2	1.0-10	2.3
59	65(O)	108	1/3sec.	2.5	0.8-15.4	2.1
49	58(A)	33	1/4sec.	2.6	1.1-8.0	1.1
41	75(O)	44	1/5sec.	1.9	0.9-3.6	0.6
61	-	74	1/9sec.	3.2	1.0-8.5	1.4
68	93(A)	71	1/10sec.	2.1	0.3-5.9	1.4
65	78(A)	30	1/15sec.	2.0	1.2-4.2	1.1
83	57(A)	23	1/15sec.	2.6	1.4-5.8	1.0

O - Orthodromic action potential; A - Antidromic action potential.

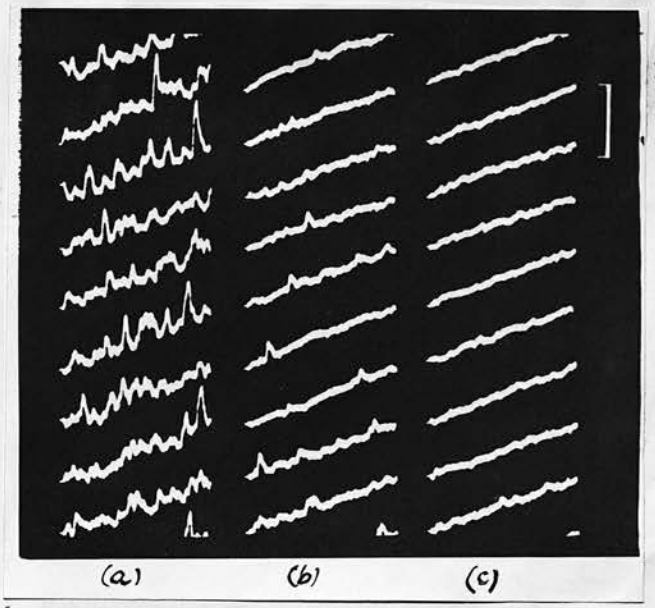


Fig. 24. Effect of tubocurarine on the miniature synaptic potentials. The external concentration of potassium was raised to 10 mM before the record (a) was taken to induce miniature synaptic potentials. Tubocurarine (to 7.5×10^{-4} M) added between (a) and (b). Voltage calibration 5 mV, trace length 550 ms. *Records read from left to right and from below upwards.*

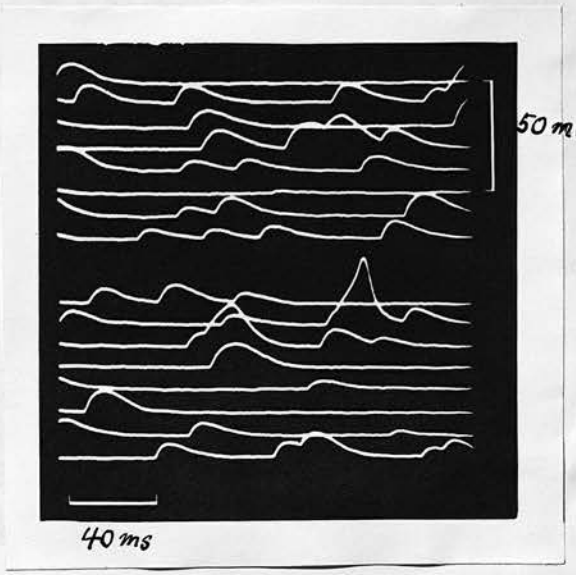


Fig. 25. Miniature synaptic potentials induced by a raised external concentration of potassium (11.8 mM). Note an action potential has been generated by the almost simultaneous occurrence of several miniature synaptic potentials. *Cell 135 (cf. Fig. 16).*

'miniature synaptic potentials' varied from cell to cell. In a majority of cells, no miniature synaptic potentials were seen during periods of observation of two to three minutes (the frequency in these cells was not likely to be greater than about 1 in 30 sec. (see Pearson and Hartley, 1958, p. 203). The highest frequency observed in normal solutions was about 20 per sec., but in only six out of more than a hundred cells was the frequency greater than 1 in 3 sec.

The intervals between successive miniature synaptic potentials appeared to be randomly distributed (Fig. 26).

Amplitude.- The mean amplitudes of the miniature synaptic potentials varied from cell to cell between 1 and 6 mV.

In a few cells, the frequency distribution of the amplitude was fairly symmetrical like that of miniature end-plate potentials, the standard deviation varying from about a third to a half of the mean. In other cells, however, the distribution was noticeably skew (e.g. Fig. 27), large miniatures (in one cell up to six times the mean value; see Table VI) being observed. In some cases their amplitude may have been exaggerated by local responses (e.g. Fig. 27A). This was indicated by the fact that they

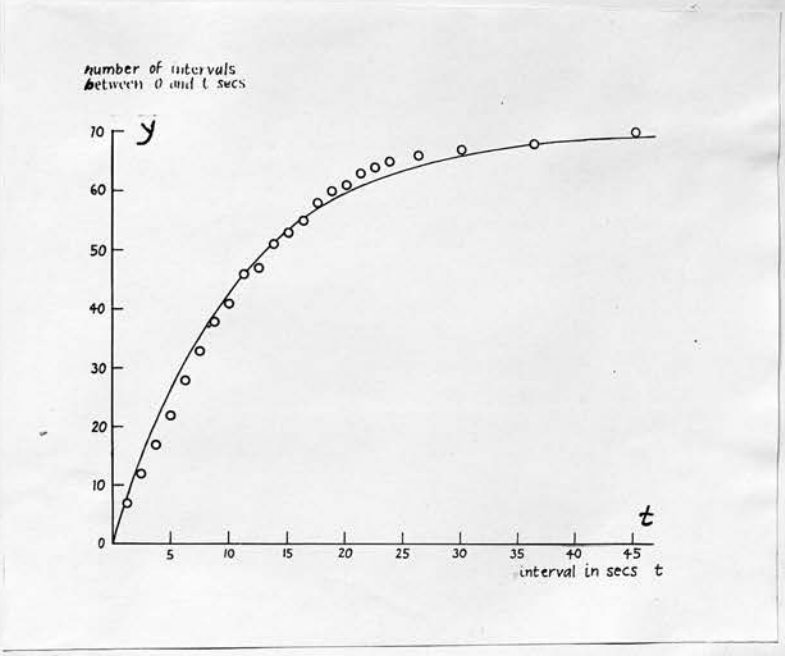


Fig. 26. Random nature of sequence of miniature synaptic potentials. Cell 68. Abcissa: t, intervals between successive miniature synaptic potentials. Ordinate: y, number of intervals less than t. Curve drawn according to the equation $y = N (1 - e^{-t/T})$, where N is the total number of intervals and T is the mean interval.

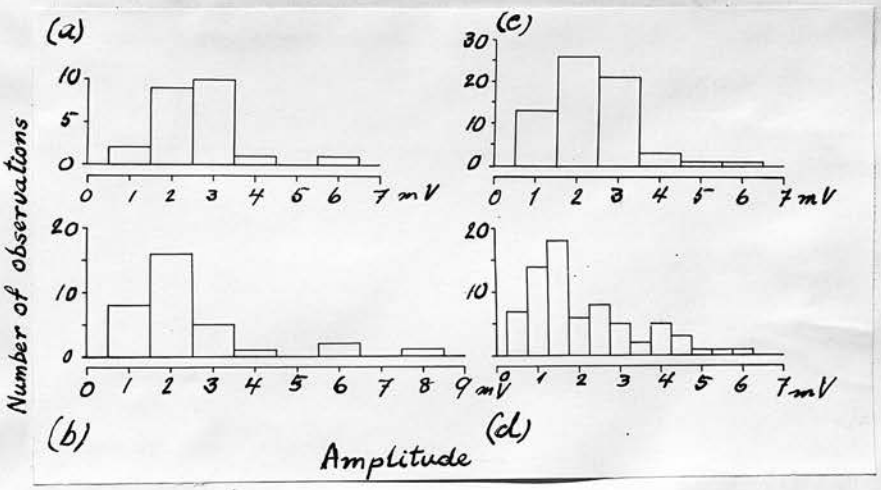


Fig. 27. Amplitude distribution of miniature synaptic potentials from four cells.

(a) Cell 83 (c) Cell 113

(b) Cell 49 (d) Cell 68

(cf. Fig. 23 and Table VI.)

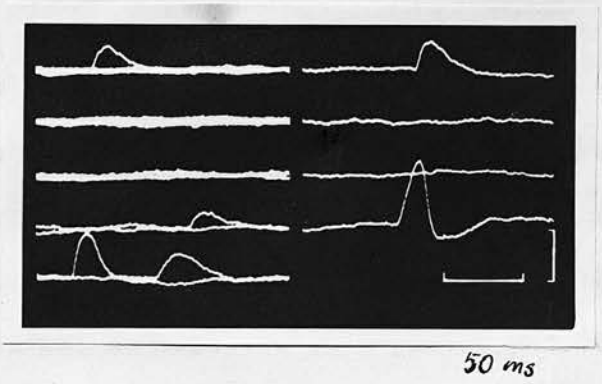


Fig. 27A. Miniature synaptic potentials.
Cell 100, Ringer's fluid with 23 mM Mg^{++}.
The very large miniature synaptic potentials have a faster rate of fall, and sometimes a 'positive phase' due to a 'local response'.

decayed more rapidly than the smaller 'miniature synaptic potentials' and had a 'positive phase' following them. However this cannot have been the sole reason for the asymmetry of the distribution since such an asymmetry was observed in cases where the amplitudes were too small to have evoked local responses. Evidently one possibility is that the quanta of acetylcholine vary greatly in size, unlike the situation at the neuromuscular junction. Another possibility is that the larger amplitudes result from the simultaneous release of a number of quanta. This would not be expected on a purely random basis, since the frequency was low; the chance emergence of two of the quanta or 'packets' of acetylcholine in a period of 10 ms, say, for a mean recurrence frequency of 1 in 3 sec, would have a probability of 1 in 300; the probability for two intervals to occur within 10 ms (i.e. the emergence of three quanta) would be 1 in 900,000. Since about a tenth of the population were larger than expected on a symmetrical distribution, some interaction at short intervals may have occurred (cf. Liley, 1956).

Time course and the conductance change underlying miniature synaptic potentials.- The rise time of the miniature synaptic potentials was of the order of 10 to 15 ms (rarely up to 20 ms) and they decayed

to half their amplitude in about 20 to 30 ms. There did not appear to be any obvious relationship between the time course and the amplitude, except in those cases where 'local response' could be detected, but in general the accuracy of measurement was limited by the slow speed of recording that was employed and by the high 'noise' level. In one cell however, it could be clearly seen that the smaller 'miniature synaptic potentials' were not slower in time course than the larger 'miniature synaptic potentials' (Fig. 25): in fact, often the larger ones took longer to rise to the peak. In this cell at least, the variation in amplitude observed (see Fig. 28) cannot have been due to varying degrees of spatial attenuation of an underlying potential change intrinsically less variable.

The maximum rate of rise of the mean miniature synaptic potentials varied in different cells from 0.56 to 2 V/sec. (see Table VII). The mean conductance change underlying the miniature synaptic potential was 0.01×10^{-6} mho.

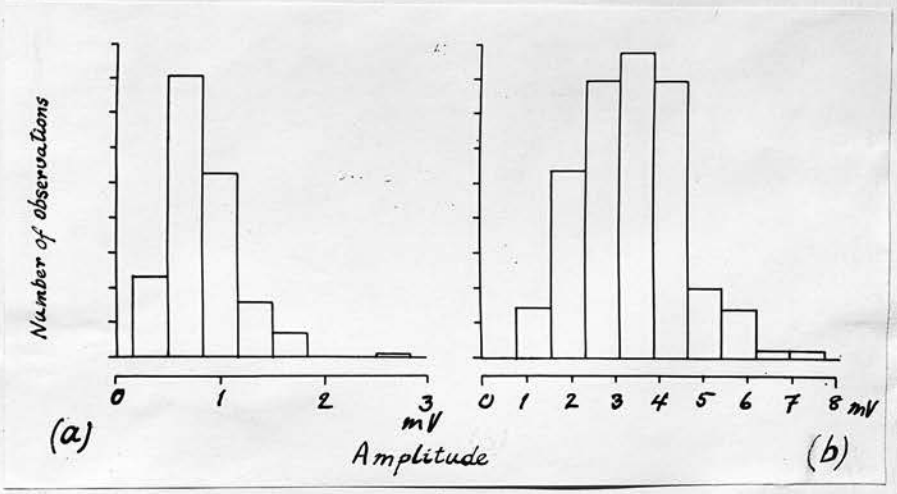


Fig. 28. Amplitude distribution of miniature synaptic potentials induced by raised external potassium concentration in two cells.
(a) Cell 118: N = 181, mean=0.83 ± 0.35 mV.
(b) Cell 135: N = 177, mean = 6.3 ± 2.9 mV.

TABLE VII

The conductance changes underlying the miniature and evoked synaptic potentials and the quantal content of the evoked synaptic potential.

Cell No.	Maximum rate of rise of the mean miniature synaptic potential V/sec	Conductance change underlying the mean miniature synaptic potential x 10^{-6} mho	Maximum rate of rise of the synaptic step V/sec	Conductance change underlying the synaptic step x 10^{-6} mho	Quantal content of the synaptic step
59	0.56	0.005	6.75	0.06	12
71	0.66	0.007	19.8	0.22	30
81	2.03	0.018	12.5	0.11	6
99	0.5	0.007	17.3	0.23	34
100	0.7	0.007	18.2	0.19	26
135	1.2	0.015	14.9	0.33	17

The conductance change, $g = \frac{C}{E} \cdot \frac{dV}{dt}$, where C is the membrane capacity, E the acetylcholine equilibrium potential (measured from the resting base-line) and $\frac{dV}{dt}$ the maximum rate of rise of the induced depolarization (at the moment of initiation - see Part I, section 2, cf. Fig. 11). The numerical value of C has been assumed to be 0.5 nV (Nishi and Koketsu, 1960) and the value of E as the estimated resting potential - 10 mV.

The quantal content of the synaptic step has been calculated from the ratio of the rates of rise of the evoked synaptic response and the mean miniature synaptic potential.

2. Factors modifying the frequency of the miniature synaptic potentials

Several procedures which are known to increase the frequency of occurrence of the miniature end-plate potentials at the vertebrate neuromuscular junction (Fatt and Katz, 1952) acted similarly on the miniature synaptic potentials in the sympathetic ganglion cells of the frog. For example, an increase in the tonicity of the Ringer's fluid caused an increase in the recurrence frequency. It was also observed in several cells that trains of rapid pre-ganglionic stimulation produced a transient burst of the miniature synaptic potentials. The most interesting phenomenon however, was the striking increase in the recurrence frequency on increasing the external concentration of potassium.

Effects of potassium.- When the external concentration of potassium was raised from its normal value of 2 mM to about 10 to 12 mM, miniature synaptic potentials were invariably seen whether or not they had been present previously (e.g. Fig. 25). The concentration required to produce an obvious increase in frequency was always in excess of that required to block presynaptic transmission. The block is most easily explained by supposing that de-polarization of the presynaptic nerves has taken

place (see Part I, section 3) and it therefore seems reasonable to assume that the increased recurrence frequency caused by the increased concentration of potassium is due to the depolarization of the pre-synaptic terminals. In several cells, in 10 to 14 mM of potassium, the recurrence frequency of the miniature synaptic potentials varied from 1 per sec. (in this cell, two miniature synaptic potentials were seen in a period of 87 sec. before the addition of potassium) to a frequency greater than 50 per sec. Since the recurrence frequency of the miniature synaptic potentials was very low before the application of potassium, it is difficult to make a reliable comparison of the amplitudes before and after potassium. Some indication however is given by the results from one cell, in which seven miniature synaptic potentials with a mean amplitude of 2.4 mV were seen before the application of potassium; after the application of K^+ to 10.4 mM the mean amplitude was 1.5 mV. A small reduction in amplitude might be expected on two counts, namely i) the reduction in the transmitter equilibrium potential due to the depolarization of the post-synaptic cell (of the order of 10 mV, see Part I, section 3), and ii) a reduction in the cell resistance (see Methods, Part II).

Figure 28 illustrates the distribution of amplitudes from two cells in 12 mM potassium.

It was unfortunately not possible to make a detailed study of the relationship between the external potassium concentration, and hence the depolarization, and the recurrence frequency. However, it was possible to show that the relationship between the depolarization and the recurrence frequency was, as at the neuromuscular junction, extremely steep. Figure 30 shows the time course of development of the effect of raising the external concentration of potassium in one cell. The recurrence frequency increased gradually over a period of 45 sec., starting about two minutes after the addition of potassium to the bath. From the results described in Part I (section 3 - see Fig. 14) it is evident that the major change in concentration around the cell occurs within 10 to 20 sec. and therefore during the period illustrated in Fig. 30, the concentration will have almost attained its final level and will have been increasing over only a very narrow range. Nevertheless, during this period the recurrence frequency of the miniature synaptic potentials increased by a factor of at least 10. An alternative explanation of these results might be that there is a delay between the onset of depolarization and the increase in recurrence frequency. This explanation may however be discounted by the results of ~~the~~ experiments in which the concentration was raised to

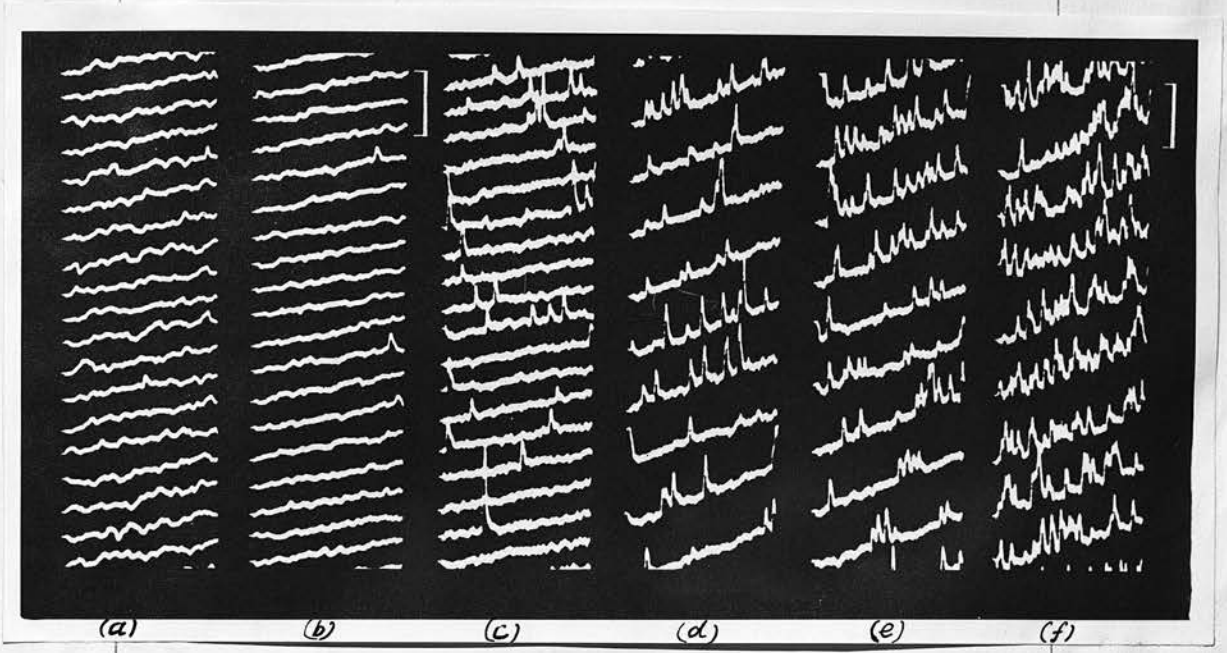


Fig. 29. Time course of effect of increasing the external K^+ concentration on the recurrence frequency of miniature synaptic potentials. Cell 87. The external K^+ concentration was raised to 10.4 mM between (a) and (b). Voltage calibration 15 mV for (a) and (b), and 5 mV for the remaining groups of traces. Trace length 570 ms. *Records read from bottom upwards. Time at which (c) to (f) were recorded are shown in Fig. 30.*

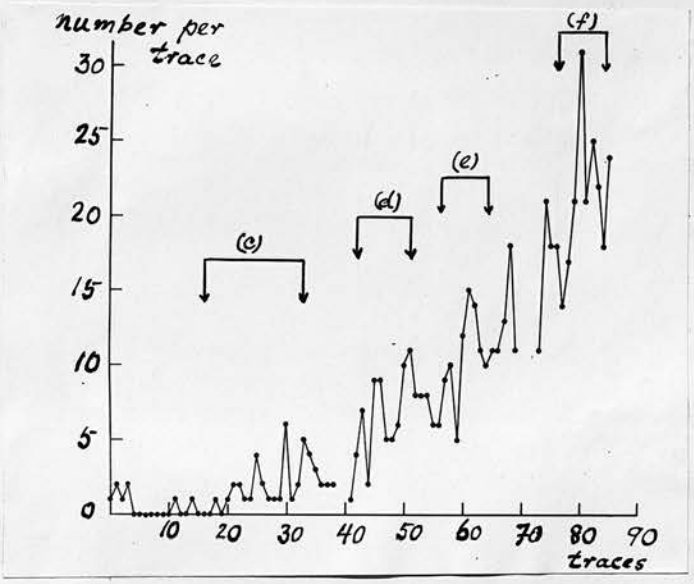


Fig. 30. The time-course of development of the effect of raised external K^+ concentration on the recurrence frequency of miniature synaptic potentials in cell 87 (cf. Fig. 29). The number of miniature synaptic potentials per trace have been shown against each successive trace. Each trace represents 570 ms. Record starts 2 min. after addition of potassium.

higher levels. In one cell, an obvious increase occurred within 25 sec. of raising the K^+ concentration to 14 mM. This period would presumably be required for the major part of the depolarization of the presynaptic terminals to occur by virtue of a 'diffusion delay'.

The increase in recurrence frequency induced by increasing the external concentration of K^+ could be reversed by adding Mg^{2+} to the Ringer's fluid. For example, in one cell, six miniature synaptic potentials were seen in a period of 52 sec.; on raising the concentration of potassium in the Ringer's fluid to 14 mM, the recurrence frequency was increased, 85 miniature synaptic potentials being seen in a period of 15 sec. On adding 18 mM/l of magnesium, only three miniature synaptic potentials were seen in the period of two minutes immediately following the addition of Mg^{2+}.

DISCUSSION

The evidence presented points to the close analogy between the miniature synaptic potentials observed in the cells of the sympathetic ganglion of the frog and the miniature end-plate potentials seen at the vertebrate neuromuscular junction. The miniature synaptic potentials are very probably the result of packets' of acetylcholine released from the presynaptic terminals.

The amplitudes of the miniature synaptic potentials are greater than those of miniature end plate potentials. This may be a reflection of the greater resistance of the cell membrane of the ganglion cell (ca. 20 MΩ, Nishi and Koketsu, 1960) as compared with the muscle fibre (ca. 0.5 MΩ, Katz and Thesleff, 1957). It is in fact tempting to suppose that the packets of acetylcholine at the synapse contain a similar number of molecules to those at the neuromuscular junction. This view is suggested by the fact that the average conductance change underlying the two varieties of miniature potential (see Table VII and Katz and Thesleff, 1957) is similar.

At the neuromuscular junction, the evoked end-plate potential has been shown to be due to the simultaneous occurrence of a number of miniature end-plate potentials. In Part III, it

will be shown that the synaptic potential is also 'quantized'. It is of interest to estimate the number of miniature synaptic potentials which make up a normal evoked synaptic potential. This may be done by comparing the rates of rise of the miniature with the evoked synaptic potential. Table VII shows the results of such calculations for several cells; the number of quanta appeared to vary between 6 and 34, and is considerably less than at the neuromuscular junction (100 - 250; Martin, 1955).

Liley (1956a) has described experiments which show that there is some correlation between the number of quanta making up the end-plate potential at the neuromuscular junction in individual fibres of the diaphragm of the rat with the frequency of occurrence of the miniature end-plate potentials in each fibre. On this basis, the comparatively rare occurrence of the miniature synaptic potentials may be related to the relatively low number of such potentials that seem to make up each evoked synaptic potential.

At the neuromuscular junction, depolarization of the prejunctional nerve terminals produces an increased rate of occurrence of miniature end-plate potentials (e.g. Liley, 1956a; see also Katz, 1962). The results described in section 2 show that this holds also for the synapse.

The depolarization of the presynaptic nerve terminals in solutions containing 10 - 14 mM K^+ is unlikely to be greater than 30 mV (see Hodgkin, 1951) yet the observed recurrence frequencies in such solutions were at least 50 times greater than those in normal concentrations of K^+. It seems probable that the 'spontaneous' output of acetylcholine from the superior cervical ganglion of the cat when it is perfused with K^+ enriched solutions (Brown and Feldberg, 1936) is related to this effect.

At the neuromuscular junction, the relationship between depolarization and recurrence frequency is extremely steep. This is indicated by the fact that the frequency varies with the fourth power of the external K^+ concentration when this is greater than 10 mM. Liley has pointed out that the relationship between depolarization of the nerve terminals and the recurrence frequency of miniature end-plate potentials provides a mechanism for the simultaneous release of a large number of packets or quanta of acetylcholine by the prejunctional action potential. It is evidently conceivable that a similar mechanism operates at the sympathetic synapse.

It has been suggested that the vesicles seen in electron micrographs of the neuromuscular junctions of several species are involved in the

quantal release of the transmitter at these junctions. Such vesicles have also been seen in the sympathetic ganglion cells of the frog (de Robertis and Bennett, 1955; Taxi, 1961). It is conceivable that these vesicles are in some way connected with the miniature synaptic potentials, and are possibly the packets in which acetylcholine is sequestered till the moment of release.

PART III

I N T R O D U C T I O N

It has been shown by Liley (1956a) that at the neuromuscular junction the simultaneous release of a large number of quanta from the presynaptic nerve terminals, in response to an action potential in the motor nerve, results from the massive increase in the frequency of release of individual quanta produced by the large depolarization of the nerve terminal. In Part II of this thesis, it has been shown that at the ganglionic synapse a relatively small depolarization of the presynaptic terminals causes a striking increase in frequency of miniature synaptic potentials. Evidently, at this site also the release of acetylcholine evoked by nerve stimulation might occur as the result of the synchronous release of a large number of quanta. Experiments which test this idea are described in this chapter.

The experiments consisted in recording with intracellular electrodes as large a number as possible of evoked synaptic responses from ganglion cells in which synaptic transmission was depressed to prevent the occurrence of action potentials. As will be described under Results, the synaptic responses from any one cell were of variable amplitude. The question to be answered is: do the fluctuations

in amplitude reflect fluctuations in the number of quanta of acetylcholine emerging from the nerve terminals? The experimental methods have been described in the two preceding parts. The methods of analysis are described in an appendix (p. 71) and references to equation numbers refer to that section.

The experiments are in principle similar to those of Castillo and Katz (1954b,c) which established the quantal hypothesis at the neuromuscular junction.

RESULTS

1. Fluctuations of responses in normal Ringer

Figure 31(a) illustrates superimposed responses from a single cell to ten antidromic stimuli. It is apparent that the form of the antidromic responses is constant. In Fig. 31(b) and (c), superimposed responses to a number of maximal orthodromic stimuli in two cells are shown. The synaptic step is evidently variable and it seems reasonable to infer that the fluctuations are due to fluctuations in the output of acetylcholine. These variations in orthodromic responses were not due to multiple innervation of the cells (see Part I, section 1). The behaviour of different cells was highly variable; rarely the fluctuations in the synaptic steps were so great (as in Fig. 31b) that a proportion of stimuli failed to produce an action potential. In many cells however, fluctuations were barely detectable. The fluctuations in the orthodromic responses may be examined more conveniently after depression of transmission to prevent the occurrence of action potentials.

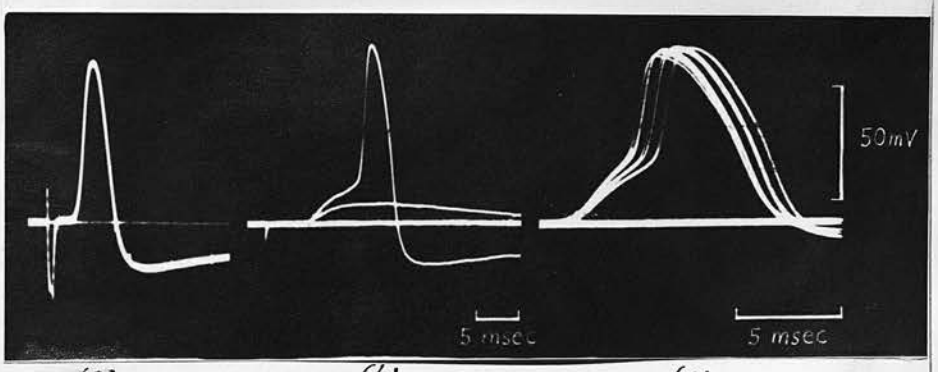


Fig. 31. Fluctuations of the synaptic step of orthodromic response in two cells (b) and (c) and absence of fluctuations of antidromic response (a). (a) Cell 57, ten superimposed responses, time calibration as in (b). (b) Cell 164, two superimposed responses. This cell had weak synaptic activity showing occasional failures to fire the cell. This effect was seen in a few cells only. (c) Cell 160, ten superimposed responses.

2. Fluctuations after depression of output of acetylcholine to low levels

Figure 32(a) illustrates a number of successive responses from a single cell to orthodromic stimuli in a ganglion bathed in a solution containing a reduced calcium concentration and to which magnesium had been added. Only synaptic potentials were observed and their amplitudes fluctuated at random. The depression in synaptic transmission is presumably at least in part due to depression of the output of acetylcholine. Such a reduction has been directly demonstrated in the cat's superior cervical ganglion (Hutter and Kostial, 1954) and also occurs at the neuromuscular junction of both amphibia and mammals (see Katz, 1962). This view is also supported by the records illustrated in Fig. 32(b) which show that as at the neuromuscular junction, a severe depression caused by magnesium may be reversed by a fast rate of stimulation. Some degree of post-synaptic depression would however also be expected since it occurs at the neuromuscular junction (Castillo and Katz, 1954a, see also Discussion). The sensitivities of different cells to low calcium or to high magnesium varied widely; in several cells for example, action potentials were observed in response to every orthodromic stimulus in as much as 20 mM magnesium. When the concentration

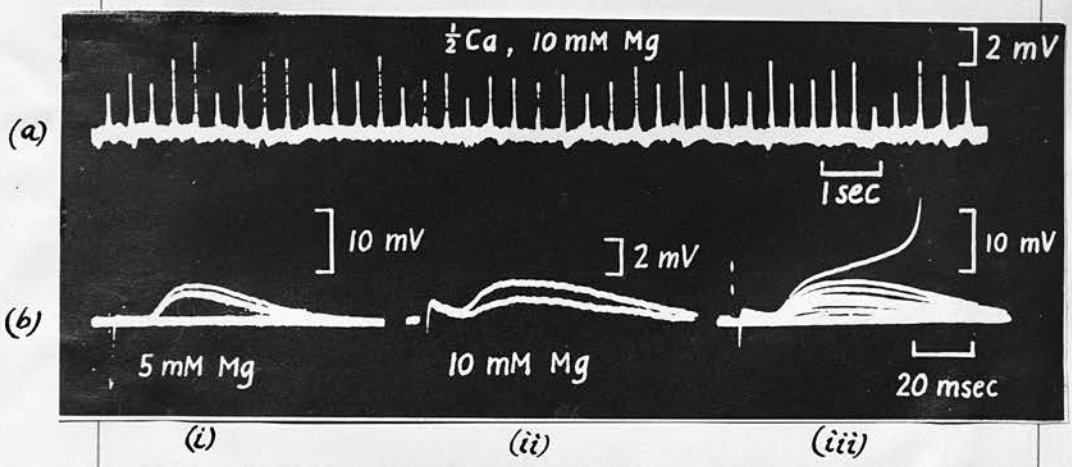


Fig. 32(a). Fluctuations of evoked synaptic potentials in low Ca^{++}/high Mg^{++} Ringer's fluid. Cell 29.

Fig. 32(b). Reversal of Mg^{++}- block by high frequency stimulation. Cell 19. Superimposed evoked synaptic potentials. (i) In 5 mM Mg^{++}. (ii) In 10 mM Mg^{++}, note increased gain. (iii) In 10 mM Mg^{++}, frequency of stimulation increased.

of calcium was sufficiently reduced or the concentration of magnesium was sufficiently increased, the fluctuations in amplitude were enhanced, occasional stimuli failing to produce any response (Fig. 33a and b). In this condition the smaller responses were similar in amplitude to the miniature synaptic potentials observed in the same cell (Fig. 34). This observation is qualitatively consistent with the idea that acetylcholine is released in the form of quanta which individually give rise to the miniature synaptic potentials and that in the presence of a high Mg/Ca concentration, the mean output per stimulus of acetylcholine is reduced to a small number of such quanta. A quantitative test of this hypothesis is outlined in the theoretical section (p. 71). As described in that section, the distribution of amplitudes of the evoked responses has been analysed on the assumption that it is based on a Poisson distribution (cf. Fig. 35), and from this analysis the mean number of quanta per stimulus and the mean amplitude of the response to a single quantum (the mean unit response) have been deduced (see equations 1 and 5). According to this hypothesis, the mean unit response, $\bar{u}$, should be equal to the mean amplitude of the observed miniature synaptic potentials. A comparison of these values is shown in columns 7 and 8 of Table VIII (in cells 69 and 72, $\bar{u}$ has been deduced from equation 6). In general the agreement is satisfactory. In

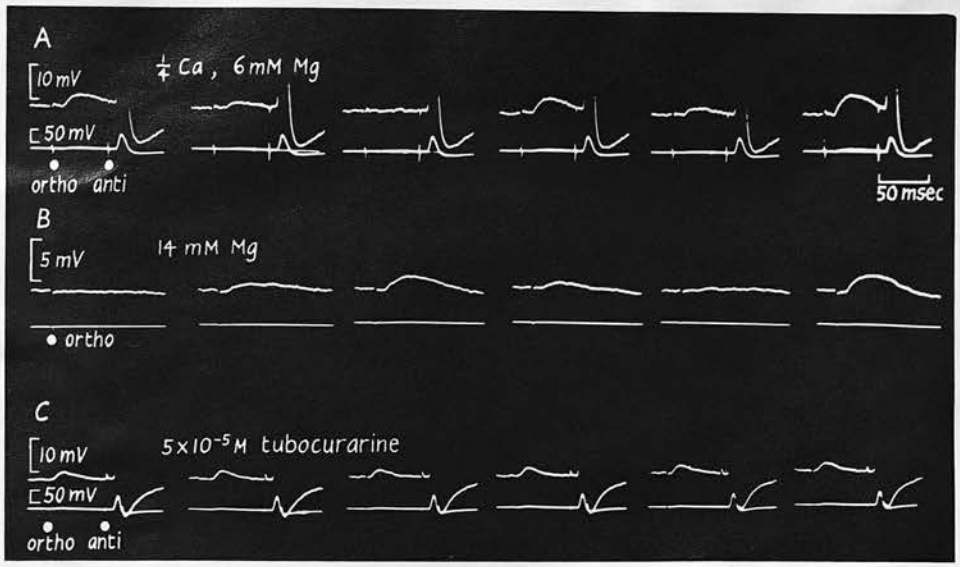


Fig. 33. Successive synaptic potentials from three cells. (A) Cell 72, (B) cell 25, (C) cell 32. Six successive responses from each cell are shown. Cells (A) and (B) in high Mg^{++} and fluctuations of synaptic potentials can be seen. Note failure of synaptic response in the third trace in (A) and in the first and the fifth traces in (B). Transmission in cell (C) was blocked with tubocurarine. The top traces in (A) and (C) were recorded at ten times the gain of the bottom traces - synaptic responses followed by antidromic responses were recorded on each trace, only the 'positive phases' of the antidromic action potentials can be seen in the top traces.

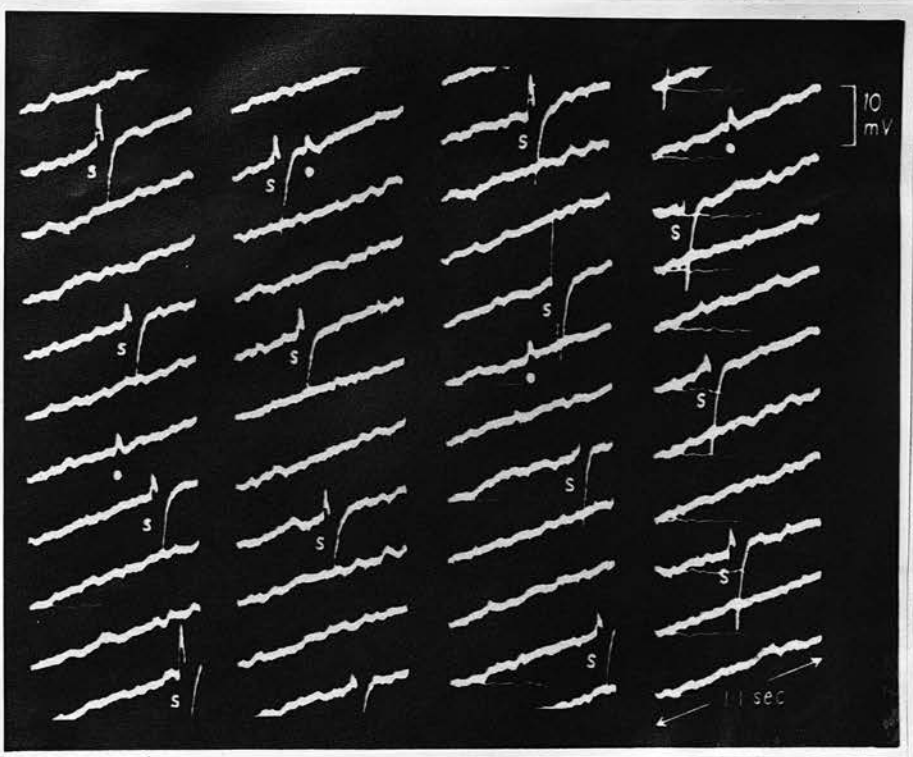


Fig. 34. Synaptic responses and miniature synaptic potentials. Cell 72 (cf. Fig. 33). Responses recorded on film moving slowly across a free-running trace. Orthodromic stimulation followed by antidromic stimulation 1/3 sec. (marked 's'). Failures can be seen in the third column (2nd from bottom) and the last column (3rd from bottom). The cell fired orthodromically (3rd column, 3rd from bottom). 20% of the total number of responses were failures.

Miniature synaptic potentials can also be seen at places marked with a 'dot'.

TABLE VIII

Comparison of the 'mean unit response' with the mean miniature synaptic potential.

Cell No. (1)	No. of stimuli (2)	No. of 'failures' (3)	'Mean quantal content' from (2) and (3) (4)	Evoked responses		'Mean unit response' from (4) and (5) (7) mV	Miniature synaptic potentials		
				Mean amplitude (5) mV	Maximum amplitude (6) mV		Mean amplitude (8) mV	Range mV (9)	No. observed (10)
25	96	32	1.10	1.10	5.2	1.0	0.93	0.7 - 1.3	12
37	207	66	1.14	1.02	3.5	0.89	0.86	0.5 - 1.6	17
69	61	7	2.16	1.90	5.6	0.88	1.0	0.6 - 1.8	9
72	100	20	1.61	3.44	12.0	2.14	1.6	0.9 - 2.7	14

view of the uncertainty in the form of the distribution of amplitudes of the response to a single quantum an unequivocal justification of the assumption that the distribution of amplitudes of evoked synaptic potentials is consistent with a Poisson distribution is not possible. The distribution of amplitudes from cell 25 has been plotted in Fig. 35. It can be seen that the observations are well fitted by a Poisson distribution if it is assumed that the amplitudes of responses to a single packet are normally distributed with a standard deviation of 34% of the mean, the mean being equal to the mean miniature synaptic potential observed. (The observed coefficient of variation was 22% but this value was derived from only 12 observations). This value for the coefficient of variation is somewhat smaller than that observed in most cells for the miniature synaptic potentials, nor are they normally distributed (see Part II), so that the agreement between the calculated and the observed curves should not be stressed. It is however evident that the general form of the observed amplitude distribution is consistent with a Poisson distribution. The distribution of the corrected amplitudes (see equation 4) in four cells were analysed in a similar way, two of which are shown in Table VIII. In the other two cells no miniature synaptic potentials were

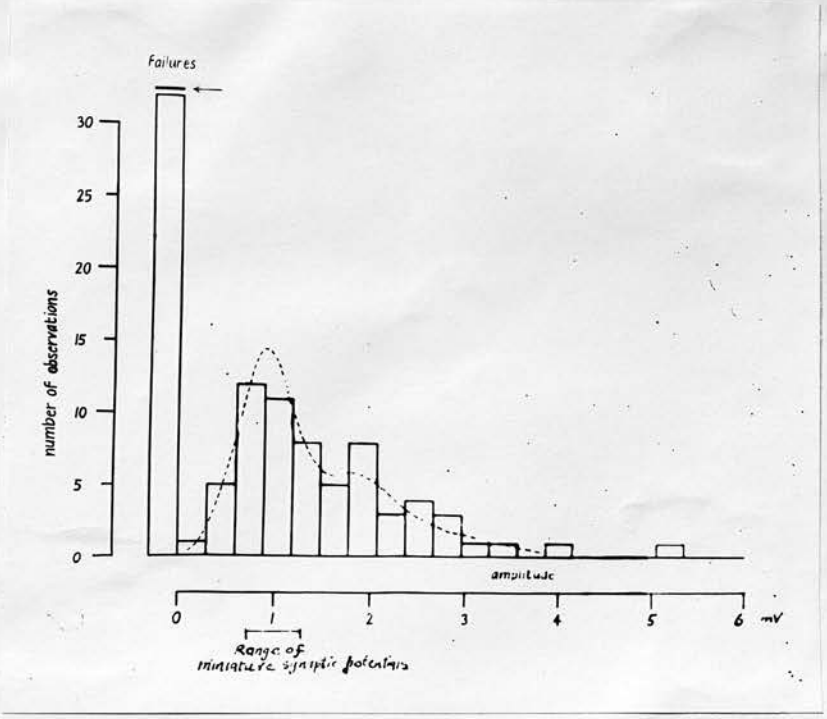


Fig. 35. Amplitude distribution of evoked responses in high Mg^{++}. Cell 25 (cf.cell B, Fig. 33). The arrow and the dotted curve indicate theoretical distribution on the assumption that the proportion of stimuli liberating different numbers of quanta follow a Poisson distribution and that the mean response to a single quantum is identical with the mean miniature synaptic potential. The dotted curve was constructed by superimposing normal distribution of relative areas given by n_1, n_2, . . . where n_1, n_2, etc. are the numbers of stimuli which would give rise to 1, 2, etc. quanta respectively, and means and variance of $\bar{u}$, σ^2; $2\bar{u}$, $2\sigma^2$; etc.(see e.g. Boyd and Martin 1956). $\bar{u}$ was taken as 0.93 mV and σ as 0.32 mV.

observed, but the amplitudes were consistent with a Poisson distribution fitted in a manner similar to that shown in Fig. 35, but based on the unit size derived from the number of failures (equation 1) in each particular experiment. The unit sizes in these two cells were 0.86 and 1.28 mV respectively (cells 29 and 36). In cell 72, 3 out of the 100 stimuli gave rise to action potentials (the height of the synaptic step was taken as the amplitude of the synaptic response). The number of larger responses was too great to conform to a Poisson distribution on the assumption of a normal distribution of the mean unit response. In this cell also the apparent size of the evoked unit response, obtained from equations 1 and 6a was significantly greater than the observed mean miniature synaptic potential (see Discussion).

3. Fluctuations after depression of output of acetylcholine at higher levels of output

Figure 36 illustrates three different distributions of amplitudes of synaptic responses from the same cell. In Fig. 36(a), the ganglion was bathed in a solution containing 25 mM magnesium and stimulated at the rate of 2/sec. In (b), the solution contained 17 mM magnesium and the stimulation rate was 1/3 sec. In (c), the concentration of magnesium was 17 mM, but the stimulation rate was 2/sec. The mean output of acetylcholine was evidently different in the three different situations and according to the quantal hypothesis this should be due to a change in the mean number of quanta released per stimulus (the 'quantal content'). The distribution of Fig. 36a has been analysed as described in the previous section, and from the mean 'response' and the number of 'failures', the mean quantal content (m) was found to be 1.14 and the apparent size of the unit, $\bar{u}$, 0.89 mV (Table VIII), in good agreement with the mean miniature synaptic potential. From the properties of the distribution as a whole (equations 5 and 6) the mean quantal content was found to be 1.47 and the mean size of the unit response was 0.7 mV. The results of Fig. 36(b) and(c) can also be analysed on the basis of the distribution as a whole (equqtions 5 and

6) and the results obtained were m = 7.01 and $\bar{u}$ = 0.62 mV for (b) and m = 14.2 and $\bar{u}$ = 0.57 mV for (c) (see Table IX). Evidently the results are consistent with the idea that an increase in the number of units is responsible for the increased output of acetylcholine. However, there is an apparent reduction in the size of the unit. A possible cause for this reduction is that the resting potential in the experiments (b) and (c) was 40 mV, whereas in (a) it was 50 mV. Had the resting potential been 50 mV in (b) and (c), the values of $\bar{u}$ would have been about 0.78 and 0.71 mV in (b) and (c) respectively.

A similar analysis was made in a second cell at two mean levels of acetylcholine output with the results given in Table IX. In this experiment the mean output increased by about 4 times without any significant change in the unit size.

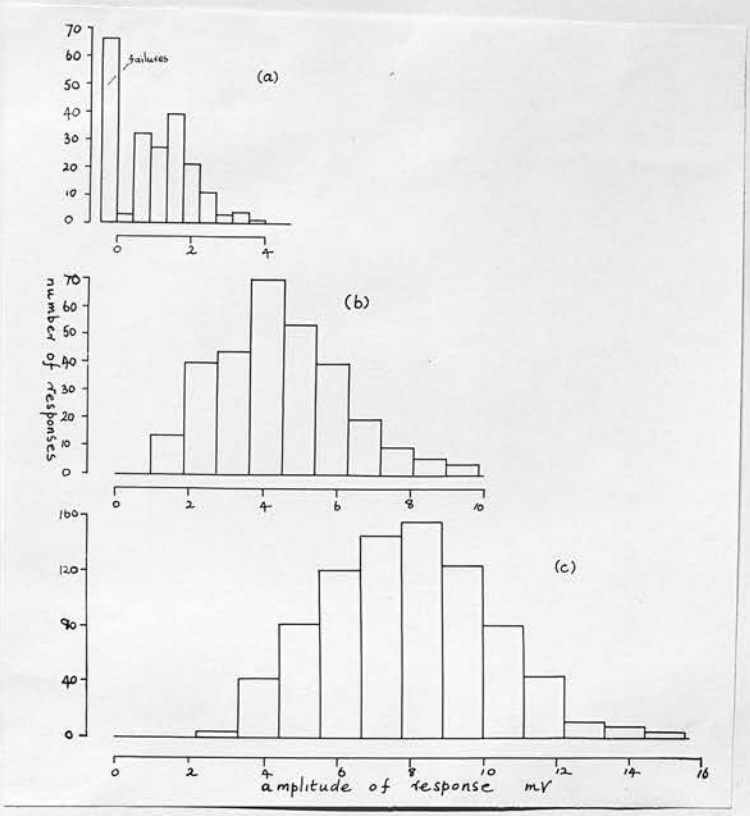


Fig. 36. Distribution of amplitudes of evoked 'responses' from cell 37, at two different frequencies of stimulation and in two different concentrations of magnesium (Table IX). (a) In Ringer's fluid containing 25 mM of magnesium, 'responses' evoked at 2/sec. (b) In Ringer's fluid containing 17 mM of magnesium, 'responses' evoked at 1/3 sec. (c) In Ringer's fluid containing 17 mM of magnesium, 'responses' evoked at 2/sec.

Table IX

Changes in quantal content with changes in mean amplitude of evoked responses.

Cell No.	Number of stimuli	Number of 'failures'	Quantal content from 'failures'	Mean response mV	Quantal content	Mean unit response from (5) and (6) mV	Mean unit response from (4) and (5) mV
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
37(a)	207	66	1.14	1.02	1.47	0.70	0.89
(b)	153	-	-	4.35	7.0	0.62(0.78)*	-
(c)	811	-	-	8.05	14.2	0.57(0.71)*	-
36(a)	161	22	2.0	2.57	2.4	1.07	1.28
(b)	97	-	-	10.8	8.6	1.25	-

Cell 37(a)	25 mM Mg^{++}	stimulation rate	2/sec.
(b)	17 mM Mg^{++}	.....	1/3sec.
(c)	17 mM Mg^{++}	.....	2/sec.
Cell 36(a)	17 mM Mg^{++}	:::.....	1/6sec.
(b)	13 mM Mg^{++}	.....	1/6sec.

* *Corrected for change in resting potential — see text, p.65.*

4. Analysis of fluctuations in the presence of curare

Figure 33(c) illustrates fluctuations in amplitudes of evoked synaptic responses when depression is produced by tubocurarine. It is clear that although the mean amplitude is not very different from the mean amplitude in the presence of reduced calcium and magnesium (Fig. 33a), the fluctuations are very much less marked, (and 'failures' were never observed in the presence of tubocurarine). The same feature is also illustrated in the distribution of amplitudes in Fig. 37. A smaller degree of fluctuation in tubocurarine is consistent with the idea that the main effect of tubocurarine is to reduce the senstivity of the cell to acetylcholine. The distribution may be analysed according to the quantal hypothesis (equations 5 and 6) and if curare exerts no effect on the output of acetylcholine, the mean quantal content should be equal to that of the normal response to nerve stimulation. Results from 3 cells are shown in Table X. In cell (a), it will be observed that the mean response was increased by trebling the external calcium concentration, an effect presumably due to an increase in the output of acetylcholine. Analysed on the basis of a Poisson distribution, on increasing the calcium concentration the quantal content rose from 26 to 64, and the unit response fell

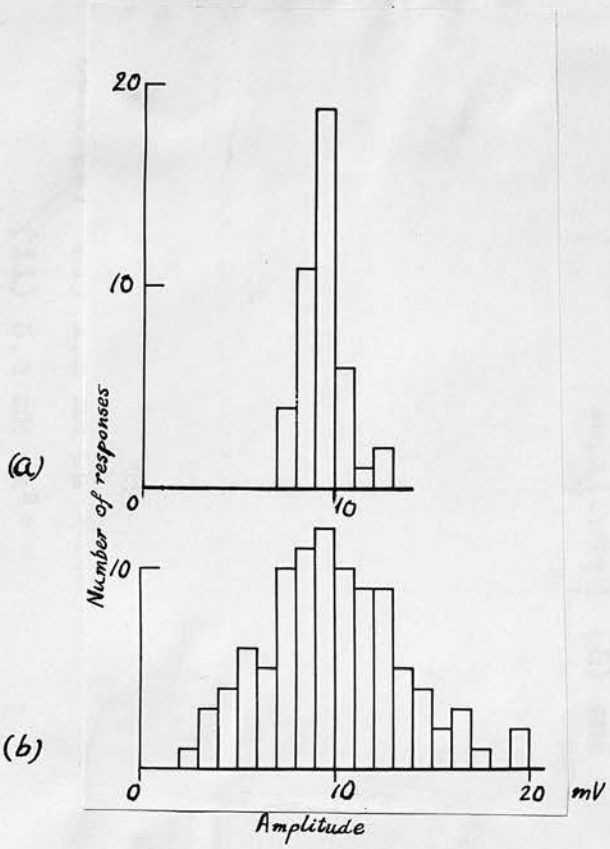


Fig. 37. Comparison between amplitude distribution of evoked 'responses' in (a) curare and (b) Mg^{++} (see Table X). (a) Cell 33, in Ringer's fluid containing 5.4 mM calcium and 5×10^{-5} M curare. (b) Cell 36, in Ringer's fluid containing a high concentration of magnesium.

Co-ordinates are the same in both diagrams.

TABLE X

Quantal content of cells in curarized ganglia.

	Evoked responses		Mean quantal content	
	Number	Mean amplitude mV	from equations (5) and (6)	from equations (5a) and (6a)
(a) i)	169	3.9	26	22
(a) ii)	3o	5.4	64	30
(b)	88	1.3	22	
(c)	43	9.4	66	

The concentration of tubocurarine in all the cells was 5×10^{-5}M.

Cell (a) (i) 1.8 mM Ca^{2+}

(ii) 5.4 mM Ca^{2+}

Cell (b) 1.8 mM Ca^{2+}

Cell (c) 5.4 mM Ca^{2+}

from about 0.15 mV to about 0.09 mV. According to the quantal hypothesis, the unit response should have remained constant. Its apparent change in size may be due to the sampling errors of the method. It may also be due to the fact that the Poisson distribution assumes an infinite latent population of quanta, and gives an exaggerated estimate for the quantal content and a reduction in the unit size if the latent population is finite (see equations 5a and 6a). The results from cell (a) would be consistent with a latent population of 48 quanta, with an average output of 22 in normal calcium, and 30 in trebled calcium. The unit size in this cell would then appear to be 0.18 mV which might be taken to indicate a five to ten-fold reduction in post-synaptic sensitivity caused by tubocurarine at a concentration of 5×10^{-5} M. No miniature synaptic potentials could be detected, nor would they be expected to be distinguishable from the base-line 'noise'.

DISCUSSION

The results which have been described are, in general, consistent with the idea that the evoked release of acetylcholine is by way of discrete quanta which may be identified with the packets of acetylcholine which give rise to the miniature synaptic potentials. In the presence of magnesium or reduced calcium, only a small number of quanta, on average, emerge from the nerve terminals in response to a stimulus. In the presence of curare, a larger number emerge but the depolarization they produce is reduced. The quantal content in curare, in two cells appeared to be about 20, and it is of interest that the normal quantal content estimated in a quite different way (Part II, p.55) varied between 6 and 34. The value obtained for the quantal content in curare is consistent both with the quantal hypothesis and the idea that the action of curare is post-synaptic.

The values obtained for the unit response in the experiments in which magnesium has been used to depress the output of acetylcholine, were in agreement with the amplitudes, in the same cells, of the miniature synaptic potentials observed, but these were somewhat smaller than those observed in most other cells in normal solution (see Part II). A reduction

in amplitude of miniature end-plate potentials to 60 to 70% of the normal value was observed by Castillo and Katz (1954a) in 16.3 mM magnesium. Several attempts were made to investigate if a similar reduction occurred at the sympathetic synapse with variable results. In two cells an increase in the concentration of magnesium to about 20 mM produced no change in the amplitude of the miniature synaptic potentials. In another cell however, a reduction in amplitude from 1.9 mV to 0.9 mV occurred on raising the magnesium concentration from 24 mM to 26 mM. Evidently, there is a great variation in the post-synaptic sensitivity to magnesium and it seems reasonable to suppose that in at least some of the cells investigated, the unit size was depressed below its normal value. On several occasions attempts were made to investigate the quantal content of evoked synaptic potentials from cells in low calcium and with a moderate magnesium concentration (as in the case of cell 72, Figs. 33a and 34, Table VIII) in which large miniature synaptic potentials were observed. It was however found that both 'failures' and action potentials occurred and measurement of the amplitudes of the large responses was made unreliable. These observations however are not necessarily inconsistent with the quantal hypothesis, since the presence of a number of unusually large miniature synaptic potentials in any one cell has

frequently been observed (see Part II, Table VI) and the simultaneous liberation of even a few correspondingly large quanta would be enough to cause an action potential.

APPENDIX

The method of analysis which was used is essentially the same as that first described by Castillo and Katz (1954b). The hypothesis to be tested is as follows: of a latent population of n packets or quanta of acetylcholine, for a fixed condition of the presynaptic terminals, a stimulus causes the release, on average, of a proportion, $\bar{p}$, of the n packets. The average number of packets, m, released per stimulus in a sufficiently long series of trials will therefore be given by

$$m = n.\bar{p}$$

In normal conditions, $\bar{p}$ may be relatively large, but by depressing the output of acetylcholine, $\bar{p}$ may be reduced. The number of packets which are released by any one stimulus cannot be predicted, but if it is assumed that $\bar{p}$ remains constant, definite proportions of a large number of stimuli can be assigned to the release of any given number of the packets.

Analysis from failures.- In particular if $\bar{p}$ is small, Poisson's Law should be followed and in a series of N stimuli, f of them, should result in the output of zero quanta, where

$$f = N.e^{-m}$$

The mean number of quanta (m) per response in a

series of N stimuli, f of which are failures should be given by

$$m = \log_e \frac{N}{f} \qquad (1)$$

Furthermore, the mean amplitude of the responses, $\bar{U}$, $(= \frac{\Sigma U}{N}$, where U is the amplitude of an individual response and may be zero) should be given by

$$\bar{U} = m.\bar{u} \qquad (2)$$

where $\bar{u}$ is the mean amplitude of the response to a single quantum. On the present hypothesis, $\bar{u}$ is equal to the mean amplitude of the miniature synaptic potentials seen in the same cell.

According to the hypothesis therefore the value of: mean response $\div \log_e \frac{\text{number of stimuli}}{\text{number of failures}}$ should be equal to the mean amplitude of the miniature synaptic potentials. This prediction has been tested in four cells with the results given in Table VIII. Obviously, this test can only be applied to a cell in which the number of failures is a significant proportion of the number of stimuli.

Analysis in the absence of failures.- If one considers the distribution as a whole, of the numbers of the packets per stimulus, then if the mean number is m, the variance of the distribution is also m. If it is now supposed, as a first approximation, that

i) each of the packets in any one cell produces a constant response u, and that ii) the responses add linearly, i.e. the response to r packets is r.u, then it may be shown that the amplitudes, U , of the responses will be distributed with a mean of $\bar{U}$, given by

$$\bar{U} = m.u. \qquad (2a)$$

and a variance of V , given by

$$V = m.u^2 \qquad (3a)$$

However, neither assumption is justified. The response produced by the packets must be regarded as variable, since the basic assumption is that they are to be identified with the miniature synaptic potentials. The effect of the variability in response to a single quantum is to modify the equations (2a) and (3a) to

$$\bar{U} = m.\bar{u} \qquad (2b)$$

$$V = m.(\bar{u}^2 + \sigma^2) \qquad (3b)$$

where, as before, $\bar{u}$ is the mean response to a single packet and σ^2 is the variance of the distribution of responses to single packets. Assumption ii) is also unjustified, since the primary effect of the action of acetylcholine on the post-synaptic membrane is a change in the conductance. If it is assumed that it is the conductance which is a linear function of the amount of acetylcholine applied, it can be

shown that the depolarization produced by a number of quanta of acetylcholine is less than the sum of the separate depolarizations. This may be allowed for approximately (Martin, 1955) by modifying equations (2b) and (3b), replacing U with

$$U' = \frac{E.U}{E - U} \qquad (4)$$

where E is the transmitter equilibrium potential (measured from the base-line), and by replacing V by V', the variance of U, in which case

$$\bar{U}' = m.\bar{u} \qquad (2c)$$

$$V' = m.(\bar{u}^2 + \sigma^2) \qquad (3c)$$

These equations have been applied to analyse the fluctuations of the responses in the absence of 'failures', since it allows the evaluation of $\bar{u}$ and m from

$$\bar{u} = \frac{V'}{\bar{U}'(1 + \frac{\sigma^2}{\bar{u}^2})} \qquad (5)$$

and

$$m = \frac{\bar{U}'^2(1 + \frac{\sigma^2}{\bar{u}^2})}{V'} \qquad (6)$$

where $\frac{\sigma^2}{\bar{u}^2}$ should be estimated from the distribution of the miniature synaptic potentials in the same cell. In practice, too few of the miniature synaptic potentials were observed to give a reliable estimate for $\frac{\sigma^2}{\bar{u}^2}$ and the value of the expression $(1 + \frac{\sigma^2}{\bar{u}^2})$

has been taken as 1.1. This value corresponds to a coefficient of variation of 33%, which is larger than that observed in the experiments to be described, but is somewhat smaller than that usually observed in normal solution (see Part II). The value of E in equation (5) has been taken as the resting potential minus 10 mV.

Analysis when probability of release is high.- When $\bar{p}$ $(= \frac{m}{n})$ is relatively large, the Poisson distribution would no longer be predicted. Equations (2c) and (3c) should then be replaced by

$$\bar{U}'' = m.\bar{u} \qquad (2d)$$

and

$$V'' = m.\bar{u}^2 (1 + \frac{\sigma^2}{\bar{u}^2})(1 - \bar{p}) \qquad (3d)$$

derived from the binomial distribution, whence

$$\bar{u} = \frac{V''}{\bar{U}''(1 + \frac{\sigma^2}{\bar{u}^2})(1 - \bar{p})} \qquad (5a)$$

$$m = \frac{\bar{U}^{2''}(1 + \frac{\sigma^2}{\bar{u}^2})(1 - \bar{p})}{V''} \qquad (6a)$$

When $\bar{p}$ approaches zero, equations (5a) and (6a) reduce to (5) and (6). If $\bar{p}$ is significantly greater than zero, it is obvious that the value derived for $\bar{u}$ from equation (5) will be reduced and that for m derived from equation (6) will be exaggerated.

Sampling errors.- The value for $\bar{u}$ and m derived from the distribution of the amplitudes of the responses will be subject to sampling errors. Unfortunately, the sampling errors of the values of m and $\bar{u}$ derived from the equations (5) and (6), and (5a) and (6a), are unknown. The variance of m as derived from equation (1) may be calculated from:

$$\text{variance } (m) = \frac{e^m - 1}{N}$$

(Fisher, 1949, p.215). The sampling error in m is smallest if the proportion of 'failures' ($= \frac{f}{N}$) is between 0.1 and 0.3 (Fisher, 1949, p.217), and for a series of 100 trials (i.e. N = 100), the standard error is then between 20 and 30%.

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serious enough to alter the main conclusions drawn in the investigations reported and were, therefore, disregarded.

Recording: In a number of experiments, the cathode follower output was led to two or three differential amplifiers connected in parallel. This was done to record responses simultaneously at different gains or at different sweep speeds. For continuous recording of long series of records, one amplifier fed a second oscilloscope (Cossor model 1049). The cathode ray tube was rotated through 90^0 so that the trace moved in the vertical plane. The records obtained on moving film were on a base line which had a slope depending on the sweep speed and the film speed. The amplitudes of responses recorded in this way could not be measured accurately, but the method had the advantage of providing a continuous record with a minimum of film.

Iontophoresis: In some experiments, acetylcholine was applied to the ganglion cells from micropipettes. The acetylcholine was ejected by positive-going square pulses applied to the inside of the micropipettes. (Nastuk, 1953; Castillo and Katz, 1955).

The micropipettes were selected from low-resistance KCl microelectrodes. Those showing resistances of 2 - 4 MΩ and able to handle relatively